MOLECULAR PATHOGENESIS OF HEPATITIS B VIRUS AND HEPATITIS C VIRUS INFECTIONS

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in Partial Fulfillment of the Requirement for the Degree
of Doctor of Philosophy in the Department of Veterinary Microbiology,
University of Saskatchewan, Saskatoon, Saskatchewan, Canada

By Qi Wu

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Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
Room C180, 105 Administration Place
Saskatoon, Saskatchewan S7N 5A2
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ABSTRACT

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections cause a wide range of liver diseases including hepatocellular carcinoma (HCC) worldwide. Because these two viruses have the same modes of transmission, HBV HCV co-infections are found in approximately 7 to 20 million people globally. HBV HCV co-infections are associated with more severe liver diseases and higher risk of HCC. Previous studies have established that HBV and HCV mono-infection can cause hepatic steatosis, and steatosis is a confirmed risk factor for HCC. However, whether and how HBV HCV co-infections synergistically increase the risk of HCC development through modulating lipid metabolism is not well understood.

PTEN is a phosphatase which contains both lipid and protein phosphatase activities. PTEN acts as a tumor suppressor by down-regulating the phosphoinositide-3-kinase (PI3K)/Akt/ sterol regulatory element-binding protein (SREBP) signaling pathway. It has been reported that PTEN is frequently mutated or deleted in tumors including HCC. PTEN-Long, a longer isoform of PTEN, which is able to be exported into the extracellular compartments, enter neighboring cells, and induce signaling events in recipient cells. Both HBV and HCV infections can inhibit PTEN and activate SREBP. However, how regulation of PTEN/SREBP pathway affects HBV and HCV infections is not fully understood. Therefore, the effect of the PTEN/SREBP pathway on HBV and/or HCV infections is worthy to study.

In this study, we showed that both HBV X protein (HBx) and HCV core protein regulate PTEN/SREBP pathway. We established that HBx activates SREBP-1a and SREBP-1c through different mechanisms. This process is involved in up-regulating of HBV enhancer I or HBV enhancer II. We demonstrated multiple mechanisms of how HBx regulates HBV replication. We also showed that HCV core interacts with PTEN and PTEN-Long which is involved in regulating of HCV life cycle. In an HBV HCV co-infection cellular model, HBx and HCV core have similar regulatory effects on the PTEN/SREBP pathway as in mono-infections. However, PTEN and SREBP differentially regulate HBV and HCV replication in HBV HCV co-infection.

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DEDICATION

This thesis is dedicated to my brilliant and lovely wife,

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Without her support and belief in me I would never have been able to accomplish this work.

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LIST OF ABBREVIATIONS

aa.	-	amino acid
AFP	-	alpha-fetoprotein
AP-1	-	activator protein 1
APC	-	anaphase promoting complex
ARF	-	alternative reading frame
ARFP	-	alternative reading frame protein
BFP	-	blue fluorescent protein
bHLH-ZIP	-	basic-helix-loop-helix-leucine zipper
b-ZIP	-	basic leucine zipper
C/EBP	-	CCAAT enhancer binding protein
cccDNA	-	covalently closed circular DNA
CD81	-	cluster of differentiation 81
CHB	-	chronic hepatitis B
CHC	-	chronic hepatitis C
CENP-C	-	centromere specific binding protein C
CLDN1	-	claudin-1
Co-IP	-	co-immunoprecipitation
CREB	-	cAMP-response element-binding protein
DMEM	-	Dulbecco's modified Eagle's medium
DNA	-	deoxyribonucleic acid
DNA-PK	-	DNA-dependent protein kinase
E4BP4	-	Adenovirus E4 promoter binding protein 4
EGFP	-	enhanced green fluorescent protein
ER	-	endoplasmic reticulum
FAK	-	focal adhesion kinase
FASN	-	fatty acid synthase
FBS	-	fetal bovine serum

FL	-	full-length
G418	-	Geneticin, a neomycin sulfate analog
GAG	-	glycosaminoglycan
GUSB	-	β-glucuronidase
HBV	-	hepatitis B virus
HBcAg	-	HBV core antigen
HBeAg	-	HBV e antigen
HBsAg	-	HBV surface antigen
HBx	-	HBV X protein
HCC	-	hepatocellular carcinoma
HCV	-	hepatitis C virus
HCVpp	-	HCV pseudo-particle
hpi	-	hours post-infection
HSPG	-	hepatocyte-associated heparin sulphate proteoglycan
$Hygro^R$	-	hygromycin resistance
IDU	-	injection drug user
IκB	-	inhibitor of NF-κB
IKK	-	IκB kinase
ILK	-	integrin-linked kinase
Insig	-	insulin induced gene
IRES	-	internal ribosomal entry site
L protein	-	large size of HBV surface protein
LDL-R	-	low density lipoprotein receptors
Luc	-	firefly luciferase
LXR	-	liver X-receptor
LXRE	-	liver X-receptor element
M protein	-	middle size of HBV surface protein
MAP	-	mitogen-activated protein
MAPK	-	mitogen-activated protein kinase
miRNA	-	microRNA
MOI	-	multiplicity of infection

mRNA	-	message RNA
mSREBP-1	-	mature sterol regulatory element-binding protein-1
mTOR	-	mammalian Target of Rapamycin
mTORC	-	mammalian Target of Rapamycin complex
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVP	-	major vault protein
NES	-	nuclear export signal
NF-κB	-	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	-	nuclear localization signal
NPC	-	nuclear pore complex
NS	-	nonstructural
NTCP	-	sodium taurocholate co-transporting polypeptide
OR	-	odds ratio
ORF	-	open reading frame
ORO	-	Oil Red O
PBD	-	PtdIns (4,5)P2-binding domain
PCR	-	polymerase chain reaction
PDK1	-	3-phosphoinositide-dependent kinase 1
PDZ	-	combining the first letters of three proteins - post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
pgRNA	-	pregenomic RNA
PI3K	-	phosphoinositide-3-kinase
PIP_2	-	phosphatidylinositol-4,5 bisphosphate
PIP_3	-	phosphatidylinositol-3,4,5 trisphosphate
PMSF	-	phenylmethylsulphonyl fluoride
PPAR	-	peroxisome proliferator-activated receptor
PRAS40	-	proline-reich Akt substrate 40 kDa
PTEN	-	phosphatase and tensin homolog deleted on chromosome 10
PTEN-L	-	PTEN-Long
rcDNA	-	relaxed circular DNA

RdRp	-	RNA-dependent RNA polymerase
RFP	-	red fluorescent protein
RIPA	-	radioimmunoprecipitation assay
rLuc	-	renilla luciferase
RNA	-	ribonucleic acid
ROS	-	reactive oxygen species
RT-PCR	-	reverse transcription-polymerase chain reaction
RTK	-	tyrosin kinase receptor
S protein	-	small size of HBV surface protein
S1P	-	site-1 protease
S2P	-	site-2 protease
S6K	-	p70 ribosomal S6 kinase
SCAP	-	SREBP-cleavage activating protein
SCID	-	severe combined immunodeficiency
SHC	-	Src-homology collagen
shRNA	-	small hairpin RNA
Sp1	-	transcription factor specificity protein 1
SRB1	-	scavenger receptor class B type I
SRE	-	sterol regulatory element
SREBP	-	sterol regulatory element-binding protein
TAP/NFX1	-	Tip-associated protein/nuclear export factor-1
TSC1/2	-	tuberous sclerosis 1/2
UPR	-	unfolded protein response
UTR	-	untranslated region
WB	-	Western blotting
WHO	-	World Health Organization
WT	-	wild-type

1.0 LITERATURE REVIEW

1.1 Hepatitis B Virus

1.1.1 Identification and molecular characteristics

In 1967, the hepatitis B virus (HBV) surface antigen (HBsAg) was first identified by Blumberg et al. [1,2]. The HBV particle was first observed by Dan et al. in 1970 [3]. These findings of HBV contribute to the development of effective surveillance tools, vaccines and HBV therapies [4]. The HBV vaccine is available which induces protective antibody response in 90% to 99% vaccinees [5]. However, the protective antibody response is not permanent, and therefore vaccination renewal is required [6].

The human HBV is an enveloped incomplete double-strand DNA virus which belongs to the family *Hepadnaviridae* [4]. The genome of HBV is unusual because the DNA is not fully double-stranded. One end of the full length strand is linked to the viral DNA polymerase. The 5'-end of the two strands of DNA contain two direct repeats (DRs) which are DR1 and DR2. DR1 and DR2 play important roles in maintaining the circular configuration and HBV viral replication [7]. The HBV DNA genome is 3.2 kb in size and contains a 3020 - 3320 nucleotides long negative strand DNA (for the full-length strand) and an 1700 - 2800 nucleotides long positive stand DNA (for the short length-strand) [8]. HBV genome encodes four highly overlapping open reading frames (ORF) which are called C, P, S and X, respectively [9]. The HBV core protein, known as HBV core antigen (HBcAg), is encoded by the C gene and produced by proteolytic processing of the precore protein. After cleavage of the N-terminal signal peptide and C-terminal arginine-rich sequence, the precore/core protein is converted to HBV e antigen (HBeAg). HBeAg can secrete into the serum which is not necessary for HBV replication but essential for chronic infection [10]. HBV P proteins are encoded by gene P. HBV P proteins are important for reconstitution of HBV DNA synthesis. Gene S has the gene that codes for the surface antigen

(HBsAg). The gene S has one long open reading frame but contains three in-frame "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small (L, M and S) are produced. A nonstructural protein, HBV X protein (HBx), is encoded by gene X which functions as a transcriptional transactivator of host genes [7] (Fig. 1.1). HBV gene transcription is regulated by two HBV enhancers: HBV enhancer I/X promoter, HBV enhancer II/core. HBx activates HBV enhancers and thus increases HBV replication [11,12].

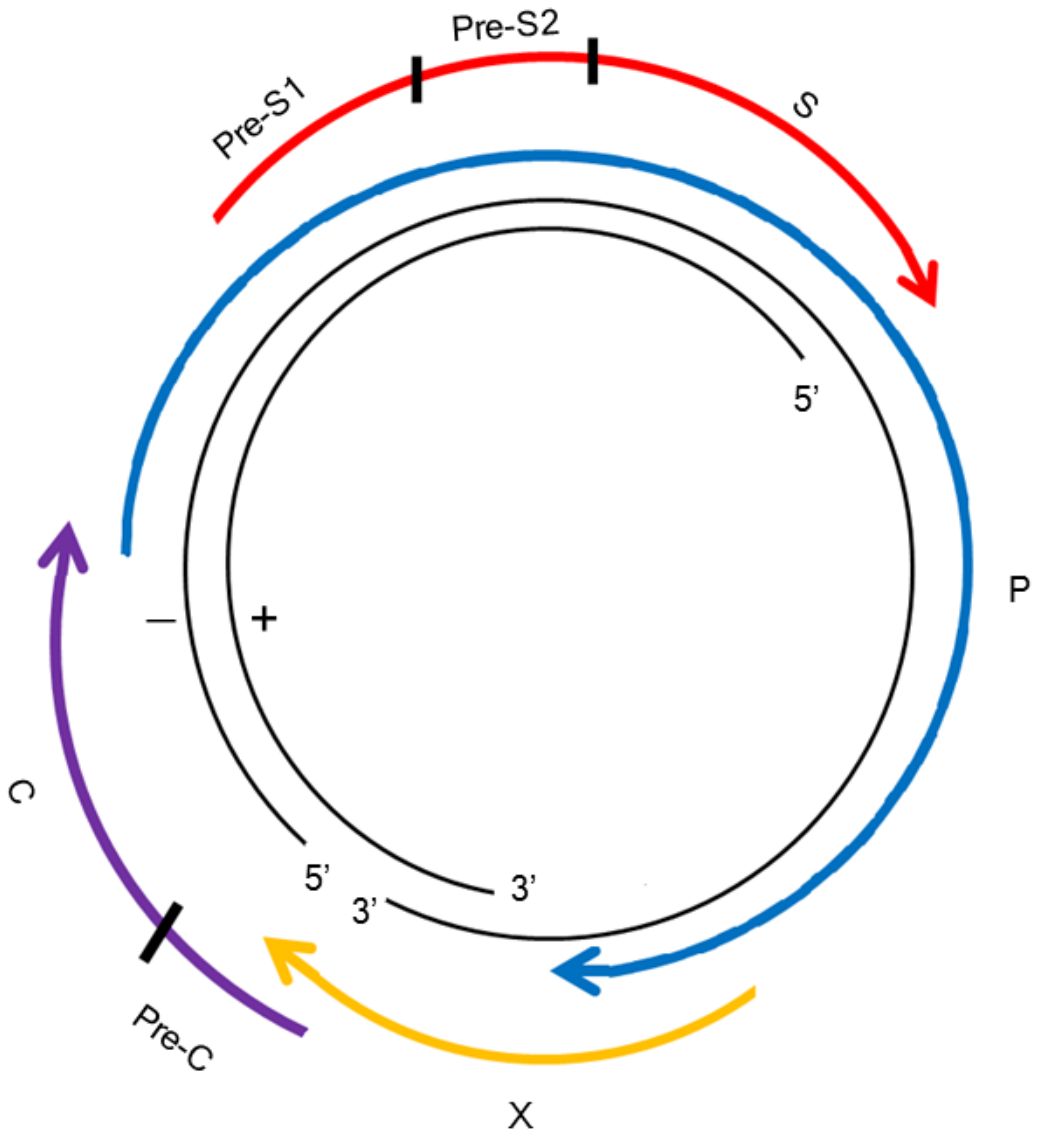

Fig. 1.1. Organization of hepatitis B virus (HBV) genome. The HBV DNA genome contains a long negative strand DNA and a short positive stand DNA. HBV proteins are encoded by four open reading frames (ORFs) which are called C, P, S and X.

1.1.2 Epidemiology

The World Health Organization (WHO) has established that there are more than 257 million chronic hepatitis B (CHB) patients [13]. HBV causes around 600,000 deaths per year and it is ranking the 15th of the causes of death [14]. HBV has been divided into 10 genotypes (A-J) (genome diversity > 8% at the nucleotide level) and further divided into more than 40 sub-genotypes (genome diversity > 4% at the nucleotide level) [15]. The prevalence of HBV varies significantly in different regions of the world. Up to 70-90% of the population are positive for serological markers in Southeast Asia, China, the Korean peninsula, sub-Saharan Africa and many Caribbean Islands. In North America and Europe, less than 2% of the population are HBV positive [16-18]. The common routes of HBV transmission include perinatal, early inapparent childhood infection, tribal tattooing and scarification, sexual contact, blood transfusions, unsafe injection practices, injecting drug use and occupational exposure of health care workers [4].

1.1.3 HBV life cycle

HBV particle entry requires host receptors hepatocyte-associated heparin sulphate proteoglycans (HSPGs) and sodium taurocholate co-transporting polypeptide (NTCP). After HBV particle uncoating, HBV releases relaxed circular DNA (rcDNA) in endosomes, rcDNA then associates with nuclear pore complex (NPC) to import into the nucleus. After the translocation, rcDNA is converted to covalently closed circular DNA (cccDNA). Then cccDNA is transcribed to pregenomic RNAs (pgRNAs) and subgenomic RNA which act as both the templates for reverse transcription to generate viral DNA and message RNAs (mRNAs) for viral protein translation after export into the cytoplasm by associating with a cellular Tip-associated protein/nuclear export factor-1 (TAP/NFX1) and HBcAg. The pgRNAs are selectively packaged inside core particles, and then followed by P protein-mediated negative strand DNA synthesis. After the degradation of pgRNAs, the positive strand DNA is synthesized to generate rcDNA. At last, HBV virions are assembled in the endoplasmic reticulum (ER)-Golgi compartment and then released from the cell. HBV rcDNA may also return to the nucleus to repeat the replication process producing more cccDNA [19,20] (Fig. 1.2).

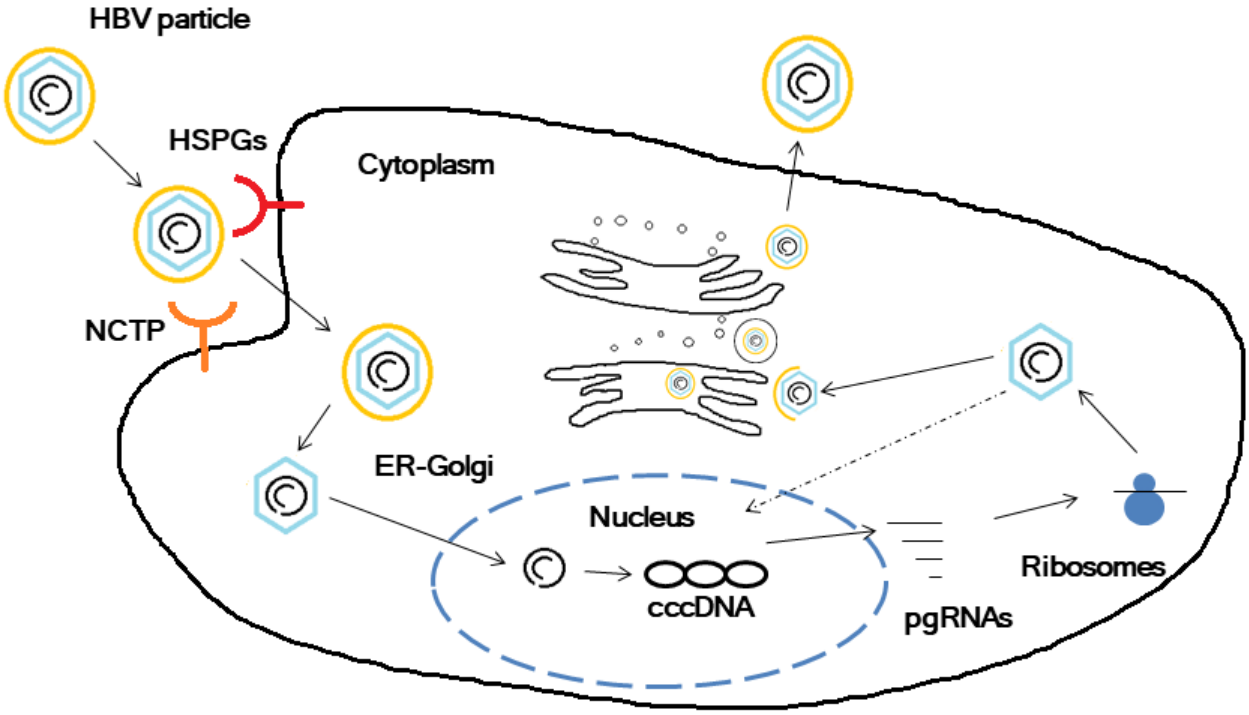

Fig. 1.2. HBV life cycle. HBV life cycle starts with HBV entry which is assocated with the host factors: hepatocyte-associated heparin sulphate proteoglycans (HSPGs) and sodium taurocholate co-transporting polypeptide (NTCP) and then followed by uncoating, and nuclear transport of the relaxed circular DNA (rcDNA). The rcDNA is converted to covalently closed circular DNA (cccDNA), which acts as the template for transcription of four RNAs. The size of the RNAs are 3.5, 2.4, 2.1 and 0.7 kb. These RNAs are exported to cytoplasm for protein translation and rcDNA generation. Then rcDNA is either enveloped in the endoplasmic reticulum (ER)-Golgi compartment and then released from the cell or transported to the nucleus to generate more cccDNA.

1.2 Hepatitis C Virus

1.2.1 Identification and molecular characteristics

The hepatitis C virus (HCV) was first identified in 1989 [21]. However, after more than 25 years of research, a vaccine to prevent HCV infection is still unavailable [22].

HCV is a small (55-65 nm in size) RNA virus of the family *Flaviviridae*. HCV is an enveloped virus with a single positive-strand RNA genome of about 9.6 kb. At the 5' and 3' ends of the genome are untranslated regions (UTRs), which are important for HCV translation and replication. Structural proteins include: core, which forms viral nucleocapsid; E1 and E2, which are envelope proteins. P7, a viroporin, is an ion channel and regulates membrane permeability and secretion. Other nonstructural proteins include NS2, NS3, NS4A, NS4B, NS5A, and NS5B. NS2, NS3 and NS4A are involved in proteolytic processing of the viral polyprotein. Two different proteinases are required for the proteolytic processing: the NS2-NS3 zinc-dependent metalloproteinase and the NS3 serine proteinase located in the N-terminal region of NS3. The C-terminal region of NS3 has RNA helicase and NTPase activities. NS4A is a cofactor of NS3 proteinase. NS4B is required for the formation of an intracellular membrane structure, the membranous web, which is essential for viral replication. NS5A is a phosphorylated protein with two phosphorylated forms: p56 and p58. One of the main functions of NS5A is RNA binding which is important for viral replication and assembly. NS5A interacts with cellular proteins and thus modulates cellular pathways associated with the immune system, cellular signaling, cell adhesion, cellular growth and death. These processes contribute to HCV pathogenesis. NS5B is the RNA-dependent RNA polymerase (RdRp) [23,24]. In 2001, a bioinformatics- and protein-based study has demonstrated that a new HCV ORF exists in the -2/+1 alternative reading frame (ARF) [25]. In the same year, another study has reported that the HCV core protein-coding sequence also expresses a 17 kDa protein, which is synthesized by ribosomal frame shift [26]. In the following studies, it has been reported that in different HCV genotypes, various lengths of proteins were synthesized from the ARF [27-32]. These proteins were named ARFP [33]. The function of ARFP in HCV life cycle is still not clear. However, they may involve in regulation of immune response of host cells, such as inhibition of interferon-α secretion [34,35] (Fig. 1.3).

1.2.2 Epidemiology

The WHO has established that approxmately 71 million patients are chronicly infected by HCV [36]. HCV is a blood borne virus. The common routes of HCV transmission include: injecting drug use through the sharing of injection equipment, the reuse or inadequate sterilization of medical equipment, and the transfusion of unscreened blood and blood products, sexual contact, being born to a mother who has HCV [14]. After HCV infection, about 15%-20% of patients will spontaneously clear the acute infections. Otherwise, patients will develop chronic HCV infections [37]. The prevalence rate of hepatocellular carcinoma (HCC) in HCV patients varies in different geographic regions. More than 70% of the HCC patients in Japan and Egypt have chronic hepatitis C (CHC), whereas the rate is much lower in Italy and Spain (40-50%), the USA (20%) and China (10%) [38,39]. HCV can be divided into seven genotypes with more than 30% nucleotide variation, and each genotype can be divided into several subgenotypes [40]. HCV genotype 1 is the most prevalent genotype globally. HCV genotype 3 causes more serious liver diseases than other genotypes [41].

1.2.3 HCV life cycle

HCV entry involves a complex series of interactions with host factors, such as cluster of differentiation 81 (CD81), scavenger receptor class B type I (SRB1), claudin 1, occludin, glycosaminoglycans (GAGs) and low density lipoprotein receptors (LDL-R). After entry to the target cell, HCV induces pH-dependent membrane fusion to release HCV RNA genome into the cytoplasm. Then the HCV genomic RNA acts as mRNA to be translated into a single large polyprotein which is further processed by host and viral proteases to generate ten viral proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The HCV core protein-coding sequence also expresses a 17 kDa protein, which is synthesized by ribosomal frame shift [26]. The ER is modified by viral and cellular factors to generate a membranous web which contains double-membrane vesicles containing HCV NS proteins, HCV RNA, ER membranes and lipid droplets. Membranous web is the major site of viral RNA amplification where HCV NS5B

protein, drives a positive-negative-positive strand RNA replicating cycle to complete HCV RNA replication. The newly synthesized RNAs are packaged into nucleocapsids and associated with the HCV glycoproteins to achieve viral maturation. The mature virions are associated with lipid droplets to release from the host cell [42,43] (Fig. 1.4).

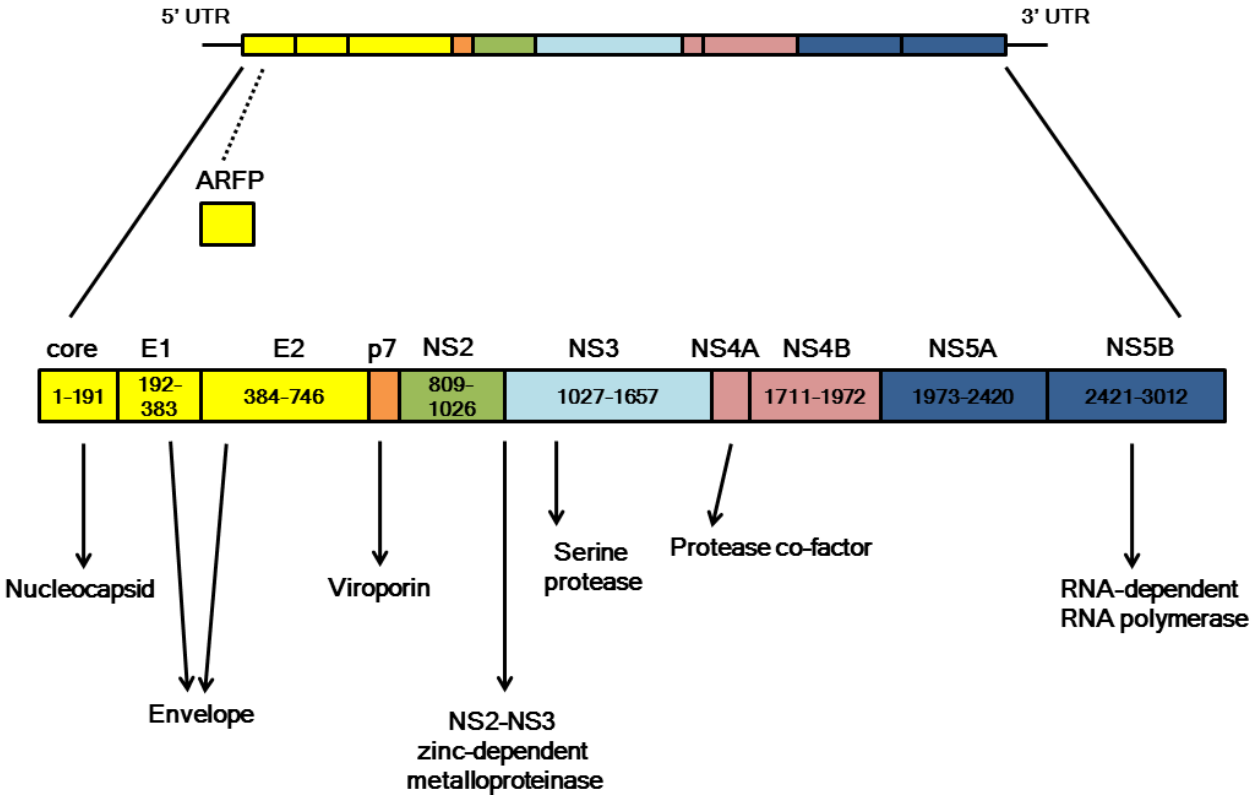

Fig. 1.3. Organization of hepatitis C virus (HCV) genome. The single positive-strand RNA HCV genome is about 9.6 kb in length. At the 5' and 3' ends of RNA exist untranslated regions (UTRs). Structural proteins include core, E1 and E2. The HCV core protein-coding sequence also expresses alternative reading frame proteins (ARFP). P7 is a viroporin. Nonstructural proteins include NS2, NS3, NS4A, NS4B, NS5A and NS5B.

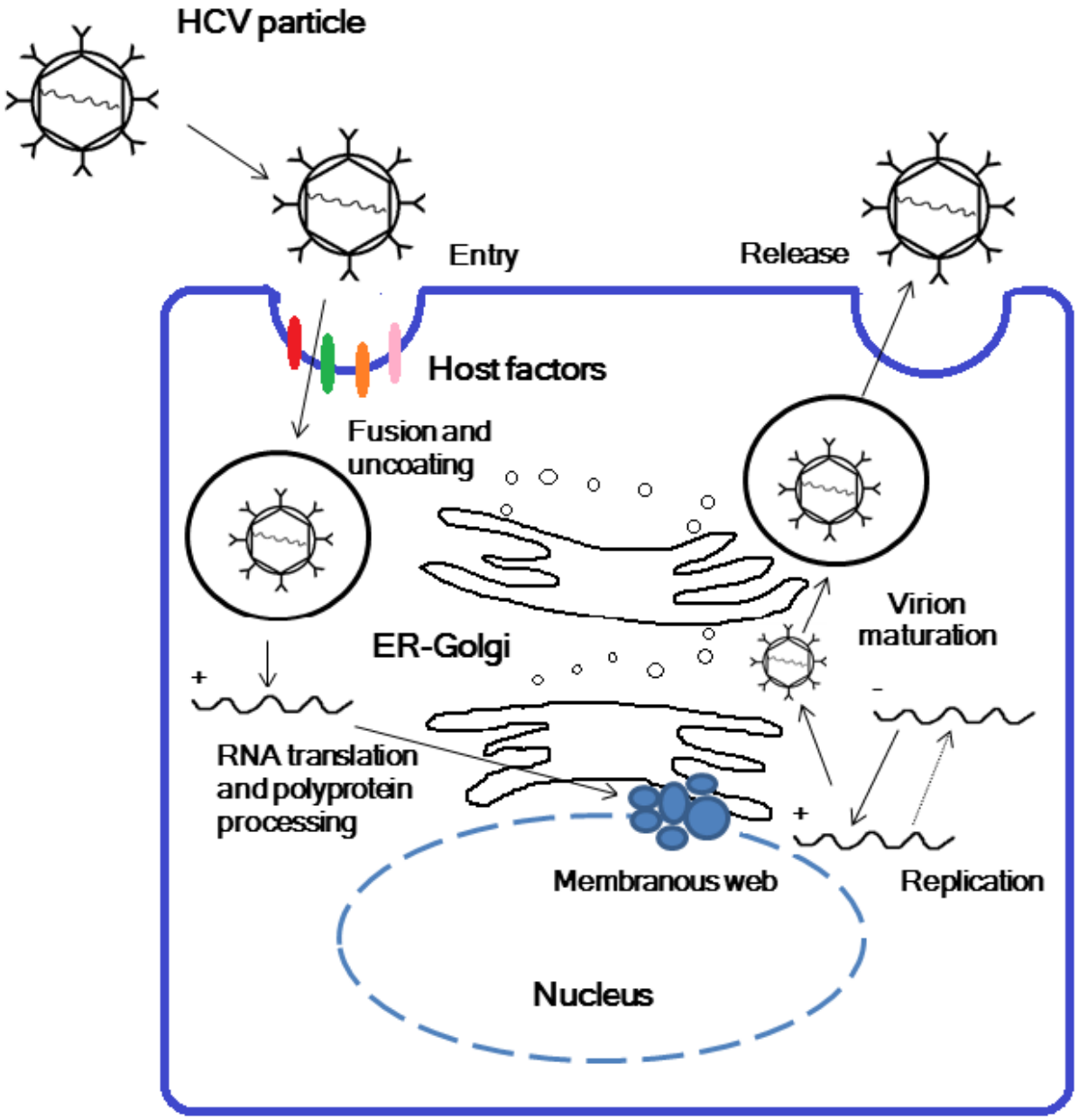

Fig. 1.4. Hepatitis C virus (HCV) life cycle. The HCV life cycle starts with HCV virions interacting with several host receptors. Then HCV glycoproteins, E1 and E2, induce pH-dependent membrane fusion and resulting in the release of nucleocapsid into the cytoplasm. The positive-strand RNA is translated to generate a single large polyprotein which is processed by host and viral proteases to generate ten mature HCV proteins. The endoplasmic reticulum (ER) is modified by viral and cellular factors to form a membranous web, where NS5B drives a positive-negative-positive strand RNA replicating cycle. Newly synthesized RNA, nucleocapsid and glycoproteins are assembled to generate new virions. Then the virions bud from the ER and achieve maturation. Mature HCV virions are associated with lipid droplets released from the cell to complete the life cycle.

1.3 PTEN Suppresses PI3K/Akt/SREBP Pathway

1.3.1 PI3K/Akt pathway

Phosphoinositide-3-kinases (PI3Ks) are members of a lipid kinase family. PI3Ks can be divided into three classes. All three classes of PI3Ks contain a C2 domain, a helical domain and a catalytic domain [44]. Class IA PI3Ks are heterodimers which contain a p110 catalytic subunit and a p85 regulatory subunit. There are three isoforms of p110, p110α, p110β and p110δ; and there are five regulatory isoforms: p85α, the splicing variants of p85α (p55α and p50α), p85β and p55γ. Class IA PI3Ks are stimulated by tyrosine kinase receptors (RTKs) or Ras and catalyze the generation of phosphatidylinositol-3,4,5 trisphosphate (PIP_3) from phosphatidylinositol-4,5 bisphosphate (PIP_2). The Class IA PI3Ks are most related to PI3K/Akt pathway [45]. Class IB PI3Ks are heterodimers which contain a p110γ catalytic subunit and regulatory isoforms p101 or p87. Class IB PI3Ks are stimulated by heterotrimeric G proteins or Ras and are essential for generation of PIP_3 in neutrophils [46]. Class II PI3Ks contain three isoforms, PI3K-C2α, PI3K-C2β and PI3K-C2γ. Class II PI3Ks are stimulated by insulin receptors, growth factor receptors and integrins. Class II PI3Ks catalyze the generation of phosphatidylinositol 4-phosphate and phosphatidylinositol 3,4-bisphosphate from phosphatidylinositol and phosphatidylinositol 4-phosphate [47]. Class III PI3Ks contain a catalytic subunit VPS34 and an adaptor subunit VPS15. Class III PI3Ks are constitutively active and catalyze the generation of phosphatidylinositol 3-phosphate from phosphatidylinositol. Class III PI3Ks regulate autophagy and endosomal protein sorting [45,48].

Akt is a serine/threonine kinase and has three isoforms (Akt1, Akt2 and Akt3). All three isoforms share approximately 80% amino acid sequence identity. Akt has an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory domain [45]. The PH domain of Akt directly binds to both PIP_2 and PIP_3 at the cell membrane [48]. Class IA PI3Ks stimulates PIP_3 accumulation, and PIP_3 recruits the PH domain, including Akt and 3-phosphoinositide-dependent kinase 1 (PDK1). PDK1 phosphorylates Akt1 on threonine 308 (Thr308), Akt2 on Thr309 or Akt3 on Thr305, and this phosphorylation of Akt results in Akt activation. The complete activation of Akt also requires phosphorylation of Akt1 on serine 473 (Ser473), Akt2 on Ser474 or Akt3 on Ser 472. These processes are regulated by PDK1, integrin-

linked kinase (ILK), an ILK-associated kinase, DNA-dependent protein kinase (DNA-PK), mammalian Target of Rapamycin complex 2 (mTORC2) and Akt itself [45,48].

1.3.2 PTEN

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase which acts on both polypeptide and phosphoinositide substrates [45]. PTEN consists of 403 amino acids and contains a short N-terminal PtdIns (4,5)P_2-binding domain (PBD), a phosphatase domain, a C-2 domain, a C-terminal tail and PDZ motif binding domain [45,49]. Two naturally occurring mutations in the phosphatase domain disrupt the phosphatase activities of PTEN: C124S mutation abrogates both lipid and protein phosphatase activities; and G129E mutation abrogates lipid phosphatase activity only [49]. The Y138L mutation, identified in 2010, abrogates protein phosphatase activity only [50] (Fig. 1.5). PTEN localizes both to the cytoplasm and nucleus. Different subcellular localizations of PTEN display various biological functions [51]. In the cytoplasm, PTEN displays both lipid and protein phosphatase activities. PTEN is considered as an essential tumor suppressor since PTEN phosphatase activity dephosphorylates PIP_3 to generate PIP_2 and thus suppresses one of the most critical cancer-promoting pathways, the PI3K/Akt pathway [45] (Fig. 1.6). Therefore, PTEN regulates various downstream functions controlled by the PI3K/Akt pathway, such as cell cycle progression, cell death, stimulation of angiogenesis and stem cell self-renewal. PTEN is frequently mutated or deleted in tumors including HCC.

Moreover, PTEN protein phosphatase activity displays several Akt-independent functions. PTEN stimulates cell migration through dephosphorylation of focal adhesion kinase (FAK) and activation of $p130^{cas}$ [52,53]. PTEN also dephosphorylates SHC by association with SHC and induces cell migration [53]. SHC is an SH2-phosphoty-rosine-binding adapter protein and recruits the Grb2-SOS complex which regulates mitogen-activated protein kinase (MAPK) [51]. In the nucleus, PTEN regulates centrosome stability through association with centromere specific binding protein C (CENP-C) [54]. Nuclear PTEN also contributes to the repair of DNA double-strand breaks through enhancement of the transcription of RAD51 [54]. In addition, cytoplasmic PTEN inhibits the activity of Akt to restore the activation of checkpoint kinase 1 which modulates the G2-M cell cycle checkpoint and thus contributes to the repair of the DNA double-

strand breaks [55]. In the nucleus, PTEN also contributes to maintaining a condensed chromatin structure through association with histone H1, C-terminus of PTEN is required for this process [56]. Nuclear PTEN down-regulates MAPK and leads to reduced cyclin D1 level and G0–G1 cell cycle arrest [57]. Moreover, nuclear PTEN modulates cellular senescence through inducing the formation of anaphase promoting complex (APC)-cadherin-1 complex [58]. Loss of nuclear PTEN is associated with more aggressive cancers [45]. A PTEN variant, PTEN-Long was identified in recent studies [59]. The start codon of PTEN-Long is located at the upstream of the coding region of PTEN. In comparison to classical PTEN, PTEN-Long contains a 173-amino acid domain at the N-terminus of the classical 403 amino acids of PTEN. PTEN-Long allows secretion in exosomes and function outside of the cell, such as regulation of Akt [59,60]. It has been reported that PTEN-Long enhances cytochrome c oxidase activity and ATP production in mitochondria [61].

Previous studies have reported that the PTEN gene is mutated in 5 % of human HCC tumors [62], and both the PTEN mRNA level and protein expression are significantly inhibited in HCC tissues in comparison to non-tumor liver tissues [63,64]. Moreover, the inhibition of PTEN expression is more frequent in higher stage HCC tumors in comparison to stage 1 HCC tumors [65,66]. These studies indicate that inhibition of PTEN expression plays a critical role in the pathogenesis of HCC development.

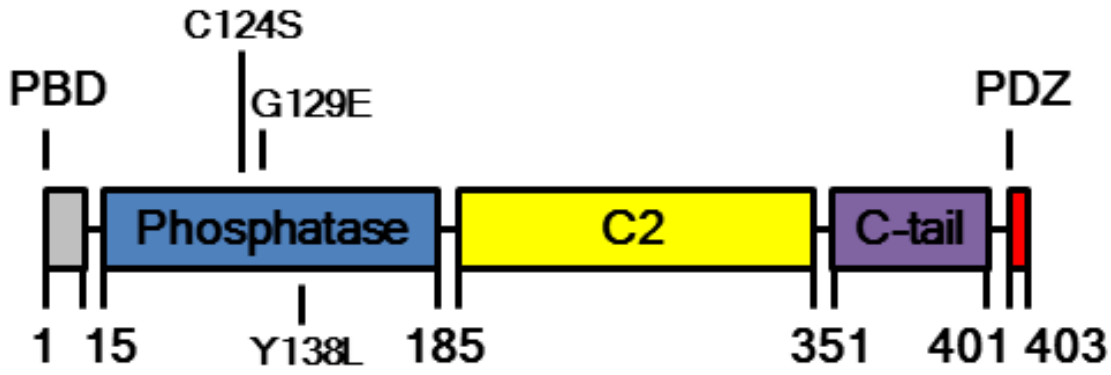

Fig. 1.5. The domain structure of phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN protein is composed of five functional domains: a PIP_2-binding domain (PBD), a phosphatase domain, a C2 domain, a C-terminal tail and a PDZ-binding domain. Three mutations in the phosphatase domain disrupt the phosphatase activities of PTEN: C124S, G129E and Y138L.

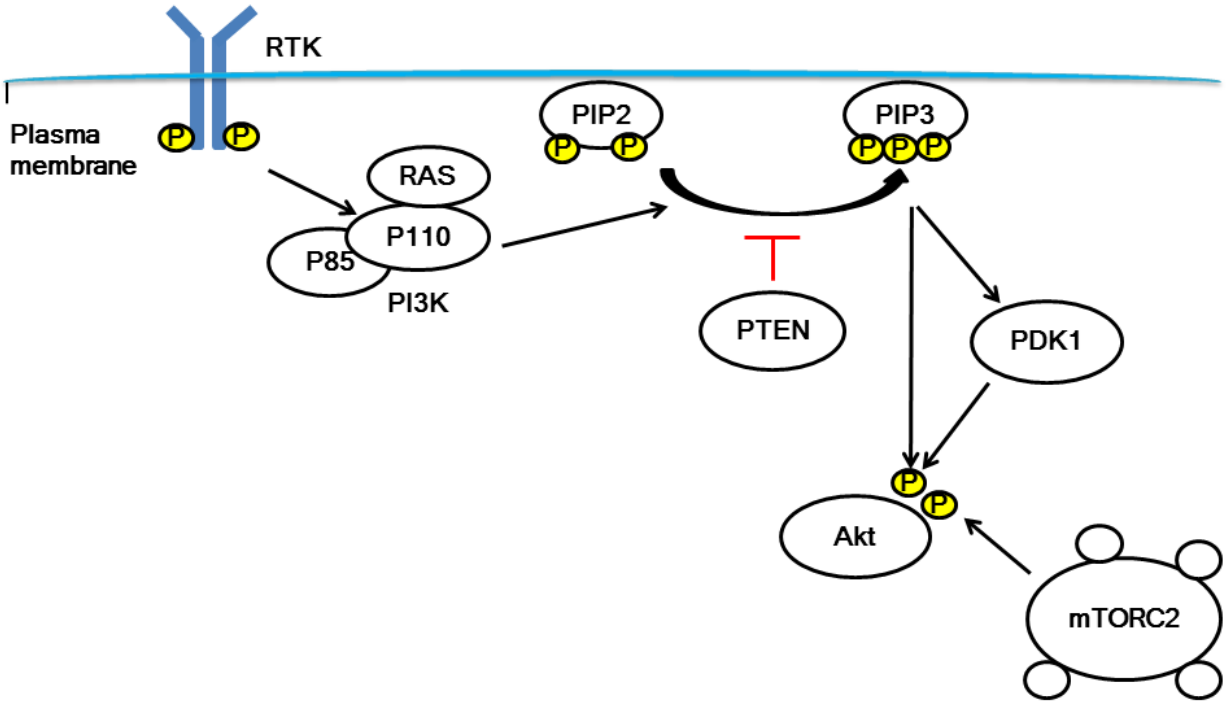

Fig. 1.6. The PTEN/PI3K/Akt pathway. Activation of phosphoinositide 3-kinase (PI3K) results in phosphatidylinositol 3,4,5-trisphosphate (PIP_3) accumulation. This process recruits the pleckstrin homology (PH) domain, including Akt and 3-phosphoinositide-dependent kinase 1 (PDK1). Once localized at the plasma membrane, Akt is activated by PDK1-mediated phosphorylation at Thr308 and phosphorylation at Ser473 by mTOR complex 2 (mTORC2). PTEN negatively regulates PI3K function, leading to inactivation of Akt.

1.3.3 SREBP

Sterol regulatory element-binding proteins (SREBPs) are transcription factors which belong to the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family [67]. Three isoforms of SREBPs, namely SREBP-1a, SREBP-1c and SREBP-2, are encoded by the mammalian genome [67,68]. SREBP-1a functions as a potent activator of all SREBP-responsive genes involving fatty acid synthesis (FASN) and cholesterol synthesis, while SREBP-1c only activates FASN but not cholesterol synthesis [69,70]. SREBP-2 is also a potent activator which is mainly involving cholesterol synthesis [71]. FASN is an essential enzyme which catalyzes de novo lipid synthesis. The abnormal activation of FASN is associated with the development of cancer [72]. SREBP-1a and -1c are encoded from the same gene. However, the promoters are different. The SREBP-2 isoform is encoded from a different gene [73]. The precursor SREBPs are synthesized as endoplasmic reticulum (ER) membrane proteins. Then precursor SREBPs form a complex with the SREBP-cleavage activating protein (SCAP) [74]. When the cholesterol concentration is low, the hexapeptide MELADL of SCAP is exposed into the cytoplasm and interacts with a COPII vesicle protein, Sec24. This process leads to the movement of the SREBP-SCAP complex from ER to Golgi apparatus [75]. In the Golgi apparatus, SREBPs are cut by two membrane-bound serine proteases, site-1 protease (S1P) and site-2 protease (S2P) [76,77]. Then, the mature SREBPs migrate to the nucleus, where SREBPs bind to sterol regulatory element (SRE) on the promoters of lipogenic genes and thus modulate lipid synthesis [78]. When the cholesterol concentration is high, a conformational change in SCAP occurs and results in the binding of the SREBP-SCAP complex to Insulin induced gene (Insig). This process inhibits SREBP activation through inhibition of SREBP-SCAP transport from ER to Golgi [79].

The activation of Akt inhibits proline-rich Akt substrate 40 kDa (PRAS40) and tuberous sclerosis 1/2 (TSC1/2) and thus induces the activation of mammalian target of rapamycin complex 1 (mTORC1) [78]. The Akt/mTORC1 pathway activates SREBP-1 through regulation of SREBP-1 expression, cleavage and nuclear localization [80]. A substrate of mTROC1, p70 ribosomal S6 kinase (S6K), plays a role in regulation of SREBP-1 expression [81]. S6K phosphorylates liver X-receptor (LXR) and stimulates the expression of SREBP-1 [82]. Activation of mTORC1 can induce ER stress and unfolded protein response (UPR) which results

in stimulation of SREBP-1 cleavage [83-85]. Moreover, activation of mTROC1 induces SREBP-1 nuclear accumulation by regulation of Linpin-1 localization [86].

1.4 HBV HCV Co-infection

1.4.1 Permission to use

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1.4.2 HBV HCV co-infection and HCC

HCC is the fifth most common cancer and the third leading cause of cancer death worldwide [87]. Infection by HBV or HCV is responsible for up to 80% of HCC cases [88]. The occurrence of HBV HCV co-infection is relatively common, particularly in areas with high prevalence of HBV or HCV infection. Generally, approximately 2–10% of anti-HCV-positive patients are HBsAg positive; on the other hand, approximately 5–20% of CHB patients are found to be anti-HCV positive. High prevalence of HBV HCV co-infection is found in some high-risk populations, such as injection drug users (IDU), patients on hemodialysis, patients undergoing organ transplantation, HIV positive individuals and beta-thalassemia patients [89-94].

One typical characteristic of cancer cells is their uncontrolled growth and proliferation in comparison to normal cells. This will definitely require more energy consumption by cancer cells and therefore it has long been recognized that abnormal lipid metabolism, especially de novo fatty acid synthesis, is a significant cause for carcinogenesis [95]. Not surprisingly, the activity of FASN, a major enzyme for fatty acid synthesis, has been found to be increased in a number of

cancers including HCC and FASN inhibitors are pursued as novel therapeutics for cancers [95-97].

1.4.3 Both HBV and HCV infections are confirmed risk factors for HCC

Transmission of HBV occurs mainly through parental, sexual and vertical (perinatal transmission) routes [16,17]. Intravenous drug use is another important route for transmitting HBV [98]. Infection of HBV causes acute and chronic liver disease, such as liver cell proliferation, steatosis, liver failure, fibrosis and cirrhosis. Moreover, HBV infection has been shown to enhance the risk for HCC 100-fold in chronic carriers. Consequently, approximately 54.4% of HCC cases are caused by HBV infection globally and as high as 89% of the HCC cases can be attributed to HBV infection in developing countries [99-102].

Hepatitis C virus is a blood-borne pathogen. Transfusion of HCV contaminated blood is the major route of transmitting HCV before implementation of HCV screening tests [103]. Therapeutic injections using contaminated needles and syringes are one important route of transmitting HCV, especially in developing countries [103]. In addition, intravenous drug use has been recognized as a major risk factor for transmitting HCV in both developing and developed countries [98,103]. HCV infection is also associated with HCC. HCV is an indirect cause of HCC due primarily to chronic tissue damage resulting from chronic inflammation, cell proliferation, steatosis and cirrhosis. It has been established that HCV infection increases the risk for HCC approximately 17-fold with approximately 31.1% of HCC cases being caused by HCV infection [100,101,104,105].

1.4.4 Steatosis associated with HBV or HCV infection is a risk factor for HCC

The term steatosis refers to the excessive accumulation of fat in the liver [106]. Clinical studies have been performed to determine a link between hepatic steatosis and CHB and CHC. A wide range of steatosis prevalence (4.5–71%) has been documented in CHB patients [107]. A mega-analysis does not suggest a direct link between CHB infection and steatosis [108]. However, clinical studies have yet to be performed to demonstrate whether HBV can cause HCC

through steatosis. Recent experimental data have shown that the X-protein of HBV can increase lipid accumulation in cell culture and cause steatosis and HCC in transgenic mouse models [109-111].

Both clinical and experimental data have demonstrated that steatosis is a risk factor for HCC in HCV-infected individuals. A clinical study of 1279 patients with CHC who received interferon therapy from 1994–2005 at a single regional hospital in Japan has shown that 68 patients (5.32%) developed HCC. It was concluded that steatosis is a significant factor for developing HCC in CHC patients, independent of other risk factors such as age, sex, obesity, fibrosis stage, and response to interferon therapy [112]. Another long-term research has also shown that hepatic steatosis is a risk factor for HCC in CHC patients [113]. This study involved 161 patients with CHC who were diagnosed at Nagasaki University Hospital, Japan, between January 1980 and December 1999. Overall, the cumulative incidence rates of HCC were 24% at 5 years, 51% at 10 years and 63% at 15 years. Steatosis developed in 90 patients (56%), and the relative risk of steatosis for HCC was 2.81, which was statistically different from the relative risk of age, cirrhosis, and interferon treatment. To understand the pathogenic effects of HCV proteins, a number of transgenic mouse lines have been generated to express either the structural proteins or the entire polyprotein [114,115]. It can be concluded that the HCV core protein itself is both steatogenic and oncogenic when expressed at high levels in mouse livers. Interestingly, when protein expression is at a very low level, only the transgenic mice expressing HCV polyprotein develop steatosis and HCC, suggesting a possible role of non-structural proteins [116]. However, the exact effects of HCV non-structural proteins on carcinogenesis are not well defined with various results being documented [117,118].

1.4.5 HBV HCV co-infection can enhance the risk of HCC

HBV HCV co-infection has been shown in numerous studies to correlate with an increased risk of HCC [93,94]. A case-control study conducted in Qidong County, an area of higher incidence of HCC in China, showed that the odds ratio (OR) values for HCC were similar in patients with the HBV (3.90) and HCV (3.89) infection, and highest in HBV HCV co-infection (6.48) [119]. Benvegnù et al. conducted a prospective study of 290 cirrhotic patients in Italy and

found that HBV HCV co-infection was an independent predictor for the development of HCC by both univariate and multivariate analyses. During a follow-up study of 8-96 months, HCC was observed in 12.2% of anti-HCV positive patients, in 19.6% of HBsAg positive patients, and in 40.0% of patients with dual positivity for HBsAg and anti-HCV [120]. A meta-analysis, conducted by Donato et al. has enrolled 32 case-control studies to investigate the impact of HBV HCV co-infection on the development of HCC [121]. This study has shown that the relative risk of HCC in co-infected patients (OR = 165) was significantly higher than HBV (OR = 22.5) and HCV (OR = 17.3) mono-infections [121].

1.4.6 Molecular mechanisms of how HBV can cause steatosis and HCC

The HBV nonstructrual protein, HBx, is considered as a transcriptional transactivator of the host genes [100]. Previous studies have determined that HBx stimulates genes that promote cell growth and inactivates growth-regulating molecules [122]. HBx contains 154 amino acids with a molecular weight of 17 kDa and it is localized in the nucleus and cytoplasm [123-125]. Different subcellular localizations of HBx display different transactivational activities. In comparison to wild-type HBx, nuclear-localized HBx significantly activates the HBV enhancer I/X but not the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or activator protein 1 (AP-1); on the other hand, cytoplasmic-localized HBx is crucial for the activation of Ras/Raf/mitogen-activated protein (MAP) kinase cascade, NF-κB and AP-1 [126,127]. A follow-up study demonstrated that amino acids 58-119 of HBx is required for activating the MAP kinase pathway [128]. Nuclear-localized HBx but not cytoplasmic-localized HBx is required for HBV replication in cell culture [129]. Previous studies have established that truncated forms of HBx are more commonly found in HBV-associated HCC tissues than non-tumor tissues [130-132]. Compared to the full-length HBx, truncated forms of HBx play different roles in HBx stability and functions. Several studies have demonstrated that the C-terminal region of HBx is essential for HBx stability [132-134]. The C-terminal region of HBx also plays important roles in stimulating HBV replication by HBx in cell culture [12,133,135]. Deletion of 23 amino acids at the C-terminal end of HBx abrogates its ability to activate NF-κB [133].

Na et al. has used transgenic mice and cell lines to show that HBx can enhance the expression of LXR and its lipogenic target genes, such as SREBP-1c, FASN and the peroxisome

proliferator-activated receptor (PPAR)-γ, which are accompanied by the lipid droplet accumulation and eventually HCC [110]. Several studies have also reported that HBx activates SREBP-1a, SREBP-1c and FASN [136,137], and this activation leads to lipid accumulation and cell proliferation in human liver cells [110,138]. Another study has shown *in vivo* and *in vitro* that HBx can enhance the transcriptional activation of SREBP-1 and the peroxisome proliferator-activated receptor-γ (PPAR-γ), and leads to hepatic steatosis [139]. Furthermore, this study has shown that HBx induces the activation of SREBP-1 through the PTEN/PI3K/Akt pathway and that HBx-induced CCAAT enhancer-binding protein C/element binding protein (EBP)-α can up-regulate PPAR-γ expression. Hye-Lin Ha and Dae-Yeul Yu have established that HBx can induce reactive oxygen species (ROS) production which can inactivate tumor suppressor PTEN and lead to live cell proliferation through Akt activation *in vivo* and *in vitro* [111]. Activation of Akt can enhance cyclin D1 expression and leads to the development of HCC [111].

1.4.7 Molecular mechanisms of how HCV can cause steatosis and HCC

The mechanisms underlying chronic HCV infection and HCC development are not well understood. It has been postulated that HCV may induce HCC by modulating lipid metabolism in hepatocytes, suggesting a link between steatosis and HCC [112,140,141]. This notion is supported by a recent demonstration of the coordinated activation of lipogenic genes in clinical HCC samples [96]. However, mechanistic studies are lacking due largely to the unavailability of an immune competent small-animal model permissive for HCV replication which can recapitulate pathology associated with both viral replication and protein expression. As such, data collected so far are from transgenic mouse and *in vitro* studies in cell culture.

The Hepatitis C virus infection of human hepatocytes transplanted into severe combined immunodeficiency (SCID), SCID/Alb-uPA mice induces lipid metabolism [142]. In addition, hepatocyte damage and apoptosis are observed, which may lead to fibrosis and cirrhosis [142]. Transgenic mouse lines expressing the HCV core protein develop hepatic steatosis followed by the appearance of HCC [115,143]. This provides direct evidence that the core protein encoded by HCV is a prominent viral factor for steatogenesis and carcinogenesis. No tissue damage is observed in transgenic mice expressing HCV envelope proteins E1 and E2, suggesting that they do not play a role [144]. Transgenic mice expressing both structural and non-structural proteins

develop abnormal lipid metabolism [116,145] and tumors [116], suggesting that HCV non-structural proteins may also play a role in steatogenesis and carcinogenesis. However, it is not clear which non-structural protein(s) are involved at this moment. Apparently, NS4B is not steatogenic and carcinogenic because NS4B transgenic mice did not show any abnormality [146]. The role of NS5A is not certain because one transgenic mouse line generated from the BCF1 strain with genotype 1b NS5A develops steatosis and liver tumors [118], whereas a genotype 1a NS5A transgenic mouse line based on the FVB strain does not show pathology [117]. Additional studies are needed to answer this question.

In vitro studies in cell culture demonstrated that HCV infection and protein expression result in abnormal lipid metabolism. Previous studies have demonstrated that both HCV core and NS5A proteins are involved in the regulation of PTEN [147-149]. A previous study has demonstrated that although the HCV 3a core does not affect the PTEN mRNA level, it inhibits PTEN expression through a 3'-UTR-dependent inhibition of PTEN translation [147]. In addition, PTEN expression is transcriptionally inhibited by the activation of NF-κB [150]. NF-κB is a group of closely related transcription factors including NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), c-Rel and RelB. NF-κB proteins can form homo- or heterodimers and display diverse transcriptional activities. While, unlike p65, c-Rel and RelB, p50 and p52 subunits of NF-κB show a lack of transactivation domain. NF-κB plays important roles in the regulation of cell apoptosis, proliferation and invasiveness, and coordination of innate and adaptive immune response [151,152]. NF-κB p65:p50 heterodimers are most common in most cell types [123]. Both p65 and p50 contain nuclear localization signals (NLS). The activation of NF-κB requires the translocation to the nucleus and binding to the target genes. However, inhibitor of NF-κB (IκB) can interact with NF-κB and retain it in the cytoplasm through either masking the NLS or exporting to the cytoplasm through strong nuclear export signals (NES) [151]. IκB can be phosphorylated on two serines sites by IκB kinase (IKK). IKK contains two catalytic subunits (IKKα and IKKβ) and the regulatory subunit (IKKγ). After phosphorylation of IκB, IκB undergoes polyubiquitination and fast degradation by proteasome which leads to translocate NF-κB into nucleus [153,154]. The HCV core can repress inhibitor NF-κB (IκB) expression and thus enhances the protein level of nuclear NF-κB. This process leads to inhibiting the protein level of PTEN and enhancing the protein level of the phosphorylated Akt [149]. Interestingly, the activation of IKK is regulated by Akt phosphorylation [155]. Therefore,

the HCV core may drive a positive feedback loop of NF-κB/PTEN/Akt and contribute to the development of HCC [150]. A previous study has reported HCV NS5A can inhibit the activity of the PTEN promoter and down-regulate the PTEN mRNA transcription and PTEN protein level. Two mechanisms are involved in this process: NS5A induces ROS-mediated NF-κB activation and NS5A enhances the activity of PI3K [156]. ROS modulates the activation of NF-κB through the inhibition of its DNA binding activity, regulation of IKK and alternative phosphorylation of IκB on Tyrosine 42 [154]. In addition, a previous study has reported that ROS can regulate the lipid phosphatase activity of PTEN through formation of a disulphide bond between Cyc71 and Cys124 [157]. These studies suggest that NS5A may also drive the positive feedback loop of NF-κB/PTEN/AKT [154-157]. Moreover, a previous study has reported a decline of nuclear PTEN protein in HCV-infected human hepatocytes [158]. PTEN does not contain a classical nuclear localization sequence but PTEN can interact with major vault protein (MVP) and translocate into the nucleus [159]. HCV infection inhibits Transportin-2 and blocks PTEN translocation into the nucleus [158]. Since the loss of nuclear PTEN is associated with more aggressive cancers [45], this study may implicate a mechanism whereby HCV infection induces HCC development.

HCV infection of hepatocytes can induce SREBP-1, SREBP-2, and their target lipogenic genes such as FASN [160,161]. Waris et al. have shown that HCV infection induces oxidative stress, inactivates PTEN by phosphorylation (HBx inactivates PTEN by oxidization) and enhances the trans-activation of SREBP through the PI3K/Akt pathway [160].

Studies from our laboratory and others have established that HCV core protein induces lipid accumulation in hepatocytes through FASN activation which can lead to the higher prevalence and severity of steatosis [162,163]. In addition, because SREBP-1 is the major transcriptional factor for modulating FASN transcription, they have also proved that the HCV core protein up-regulates the FASN promoter in an SREBP-1-dependent manner [163]. After that, they have determined that HCV core protein enhances SREBP-1 activity through the PI3K/Akt pathway [164]. Unlike HBx, HCV core protein has no effect on PPAR-γ gene expression; however, it can enhance the transcriptional activation of PPAR-γ [139]. Although PPAR-α seems not to be regulated by HBx, PPAR-α contributes to HCV core protein-induced steatosis and HCC [165].

Hepatitis C virus non-structural proteins also play a role in modulating lipid metabolism. The HCV NS2 can activate SREBP-1c and FASN transcription [166]. Moreover, the sterol

regulatory element and liver X-receptor element (LXRE) are involved in SREBP-1c activation by HCV NS2 [166]. HCV NS4B protein can activate SREBP-1 and SREBP-2 through the Akt pathway [161]. HCV NS5A protein can also activate SREBP. An *in vitro* study has shown that HCV NS5A protein can up-regulate SREBP-1c transcription through the transcription factor specificity protein 1 (Sp1), and therefore increase the level of mature SREBP-1c protein [167]. HCV NS5A has also been reported to activate the transcriptional level of PPAR-γ and induce the hepatic lipid accumulation [168].

Taken together, it can be concluded that HCV has developed multiple mechanisms for activating the host lipogenic pathway.

1.4.8 Possible mechanisms of how HBV HCV co-infections enhance the risk of HCC through steatosis

Although many studies have examined the mechanisms of how HBV or HCV mono-infections cause HCC, very few studies are directed towards the understanding of the molecular mechanisms of how HBV HCV co-infection enhances the risk of HCC. Here, we will review the available published work and provide our thoughts on future directions.

A seminal study by Dr. Slagle demonstrated that co-expressing HBx and HCV polyprotein in double transgenic mice results in more severe steatosis and an increased incidence of HCC, suggesting a synergy between HBV and HCV in pathogenesis [169]. Consistent with this finding, it has been shown that HBx and HCV core proteins can enhance cell transformation and proliferation and result in higher tumorigenicity in mice [170,171]. Additional study has shown that HBx and HCV core protein can synergistically inhibit the cyclin-dependent kinase inhibitor p21 gene and lead to cell proliferation [172].

What are the possible mechanisms for the observed synergy between HBV and HCV for steatogenesis and carcinogenesis (Fig. 1.7)? In general, activation of SREBP and abnormal activation of PPAR are two of the common reasons for abnormal lipid metabolism, and they are both are related to carcinogenesis [173-175].

Sterol regulatory element binding protein can be activated by the PI3K/Akt pathway [176]. The activation of SREBP induces the expression of FASN, and thus results in membrane lipid biosynthesis [177]. It has been demonstrated that loss or inactivation of the tumor suppressor

gene PTEN leads to the activation of the PI3K/Akt pathway. The PTEN/PI3K/Akt pathway plays a critical role in carcinogenesis [178-181]. We previously reviewed the fact that HBV and HCV protein expression as well as HBV or HCV infections have been shown to activate the PI3K/Akt pathway. Therefore, it is worth studying whether HBV and HCV co-infection may further increase the degree of activation of the PTEN/PI3K/Akt pathway, leading to more enhanced SREBP activity, more severe steatosis and eventually a higher incidence of HCC.

The UPR is another important mechanism involved in modulating SREBP activity [182]. UPR is an adaptive intracellular stress response to the accumulation of unfolded or mis-folded proteins in the ER. Activation of the UPR contributes to several kinds of liver disease, including hepatic steatosis and HCC [183-186]. ER stress can enhance the activation of SREBP [187,188]. Both HBV and HCV infection can activate UPR and lead to ER stress [185]. As such, enhanced UPR may be another mechanism responsible for the HBV and HCV synergy in causing steatosis and HCC.

Peroxisome proliferator-activated receptors are a group of nuclear receptor proteins with three members: PPAR-α, PPAR-β/δ and PPAR-γ [175]. In the liver, PPAR-α plays an important role in regulating fatty acid oxidation, lipoprotein synthesis, inflammatory responses and cancer development [175]. PPAR-β/δ acts as a plasma-free fatty acid sensor [189]. Moreover, it protects the liver by down-regulating inflammatory genes [190,191]. There are two isoforms of PPAR-γ: PPAR-γ1 and PPAR-γ2. A high level of PPAR-γ1 in mouse liver can lead to the development of hepatic steatosis [192]. As discussed previously, PPAR-α and PPAR-γ are involved in lipid accumulation and tumor formation induced by HBV and HCV. It appears that synergistic activation of PPAR activities may play an important role in increased steatogenesis and carcinogenesis in HBV and HCV co-infections.

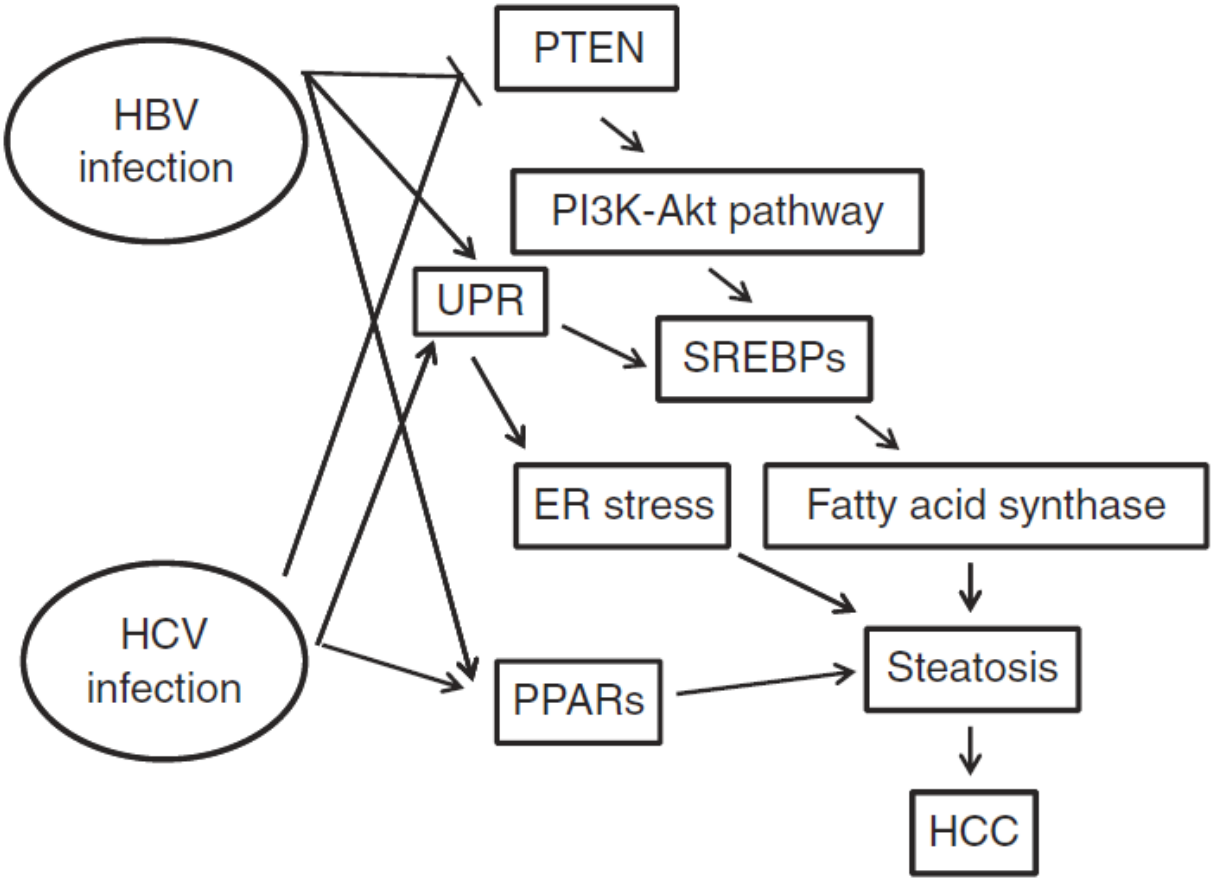

Fig. 1.7. Potential mechanisms of how HBV and HCV co-infections synergistically activate steatogenesis and carcinogenesis. ER, endoplasmic reticulum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator activatedreceptors; PTEN, phosphatase and tensin homologdeleted on chromosome 10; SREBP, sterol regulatory element binding proteins; UPR, unfolded protein response.

2.0 HYPOTHESES AND OBJECTIVES

2.1 Rationale

The term “steatosis” is used to refer to the deposition of fat in the interstitial spaces of an organ. Specifically, steatosis is commonly used to refer to depositions of fat in the liver. Steatosis is an independent and significant risk factor for HCC in chronic HBV and/or HCV infected patients. Previous studies have already established that HBV HCV co-infection enhances the risk for development of HCC. However the mechanisms are still not fully understood. Abnormal regulation of the PTEN/PI3K/Akt/SREBP pathway relates to HCC *in vitro*. Both HBV and HCV infections inhibit PTEN and thus activate the PI3K/Akt/SREBP pathway. However, the effect of PTEN and SREBP on HBV and HCV infection is not fully understood. Our research will study the potential mechanisms of how HBV or HCV mono-infection and HBV HCV co-infection enhance the activation of the lipogenic genes and leads to the higher risk for HCC. Moreover, we will also study if activation of the lipogenic genes affects HBV or HCV infection in HBV or HCV mono-infection and HBV HCV co-infection.

2.2 Hypothesis

(i) HBx may stimulate SREBP-1 activation and this process may be involved in HBx-mediated HBV replication.
(ii) HCV may inhibit PTEN which is benefit to HCV infection.
(iii) Compared to HBV or HCV mono-infection, HBV HCV co-infection may enhance hepatic steatosis and the occurrence of HCC by synergistically up-regulating lipogenic gene expression through the PTEN/SREBP pathway.

2.3 Objectives

(i) To determine how HBx regulates the activities of SREBP, FASN and HBV enhancers and how HBx affects lipid accumulation and cell proliferation.

(ii) To determine the effects of PTEN and SREBP-1a on HBV/HCV replication and to determine the effects of the PTEN/SREBP pathway on HBV/HCV induced steatosis and cell proliferation.

(iii) To determine how HBV HCV co-replication regulates the PTEN/SREBP pathway and determine the effect of the PTEN/SREBP pathway on HBV and HCV replications in HBV and HCV co-replicating cells.

3.0 SREBP-1A ACTIVATION BY HBX AND THE EFFECT ON HEPATITIS B VIRUS ENHANCER II/CORE PROMOTER

Ling Qiao [a,1,2], Qi Wu [a,2], Xinya Lu [a], Yan Zhou [a], Ana Fernández-Alvarez [b], Lihong Ye [c], Xiaodong Zhang [c], Jihong Han [c], Marta Casado [b], Qiang Liu [a,*]

[a] VIDO-InterVac, University of Saskatchewan, Saskatoon, SK, Canada S7N 5E3

[b] Instituto de Biomedicina de Valencia, Spanish Council for Scientific Research (CSIC), Valencia, Spain

[c] College of Life Sciences, Nankai University, Tianjin, China

Keywords: HBx; SREBP-1a; C/EBP; E4BP4; HBV enhancer II/core promoter; Transcription

* Corresponding author.

VIDO-InterVac

University of Saskatchewan,

120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.

Fax: +1 306 966 7478.

E-mail address: qiang.liu@usask.ca. (Q. Liu).

1 Present address: 2011 Collaborative Innovation Center of Tianjin for Medical Epigenetics, Key Laboratory of Hormones and Development (Ministry of Health), Metabolic Diseases Hospital & Tianjin Institute of Endocrinology, Tianjin Medical University, Tianjin, China.

2 These authors contributed equally to this study.

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3.2 Authors' contribution

The experiments within this chapter were equally performed by Ling Qiao and Qi Wu. The manuscript was written by Ling Qiao and edited by Qiang Liu.

3.3 Abstract

Hepatitis B virus (HBV) X protein (HBx) plays an important role in HBV pathogenesis by regulating gene expression. Sterol regulatory element binding protein-1a (SREBP-1a) is a key transcriptional factor for modulating fatty acid and cholesterol synthesis. Here we demonstrated that HBx increased mature SREBP-1a protein level in the nucleus and its activity as a transcription factor. We further showed that the up-regulation of SREBP-1a by HBx occurred at the transcriptional level after ectopic expression and in the context of HBV replication. Deletional analysis using SREBP-1a promoter revealed that the sequence from -436 to -398 in the promoter was required for its activation by HBx. This promoter region possesses the binding sequences for two basic leucine zipper (b-ZIP) transcription factors, namely C/EBP and E4BP4. Mutagenesis of the binding sequences on the SREBP-1a promoter and ectopic expression experiments demonstrated that C/EBPα enhanced SREBP-1a activation by HBx, while E4BP4 had an inhibitory effect. C/EBPα was able to significantly reverse the inhibitory activity of E4BP4 on SREBP-1a promoter. These results demonstrated that HBx activates SREBP-1a

activity at the transcription level through a complex mechanism involving two bZIP transcription factors C/EBP and E4BP4 with C/EBP being the dominant positive factor. Finally, we showed that knocking down SREBP-1 abolishes HBV enhancer II/core promoter activation by HBx.

3.4 Introduction

Sterol regulatory element-binding proteins (SREBPs) belong to the family of basic-helix–loop–helix-leucine zipper (bHLH-ZIP) transcription factors [67]. SREBPs directly activate the expression of numerous genes linked to the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. The mammalian genome encodes three SREBPs, designated SREBP-1a, SREBP-1c, and SREBP-2 [68]. In comparison to SREBP-1c, SREBP-1a has a longer transcription activation domain at its N-terminus which is capable of recruiting co-activators for transcription [68]. As such, SREBP-1a is a more potent activator of all SREBP-responsive genes for fatty acid and cholesterol synthesis, whereas SREBP-1c can only activate fatty acid synthesis [69,70]. SREBPs are synthesized as in active precursors and the N-terminal portion, released from the SREBP precursor by proteolysis, enters the nucleus and becomes active transcriptional factors [193]. Nuclear SREBPs activate transcription by binding to SRE sequence in the promoter regions of target genes [67].

Hepatitis B virus (HBV) infection is a global health problem with 350-400 million people being chronic carriers [194]. HBV has a partially double-stranded circular DNA genome coding for core, surface, polymerase, and the X (HBx) proteins [123]. Two viral enhancers promote HBV transcription [195]. Enhancer II/core promoter sequence regulates the transcription of 3.5-kb pregenomic RNA, a key step in HBV replication cycle. HBx increases HBV replication and activates HBV enhancer II/core promoter [11,196,197]. HBx also modulates host cellular functions including lipid metabolism [198,199]. In this study, we investigated activation of SREBP-1a by HBx and its involvement in regulating HBV enhancer II/core promoter by HBx.

3.5 Materials and Methods

3.5.1 Plasmids and antibodies

The coding sequence of HBx was amplified by PCR from plasmids pRBK HBx or pawy1.2 [200,201] and cloned in-frame with the myc tag into the pEF/cyto/myc vector (Invitrogen). HBV enhancer II/core promoter sequence [202] was cloned into pGL4.14 vector (Promega), generating pGL4-HBV EN2/CP where the expression of luciferase gene was controlled by HBV enhancer II/core promoter. Flag-tagged SREBP-1a (aa. 1-517) was amplified from an SREBP-1a plasmid [203] and inserted into the pCMV2 Flag vector (Sigma-Aldrich) [164]. Plasmid pSRE-Luc containing three copies of SRE sequences was provided by Shimano [204]. Human SREBP-1a promoter- luciferase reporters containing different lengths of the SREBP-1a promoter were described previously [205]. Mutant SREBP-1a promoters with mutations for the binding sequences for C/EBP (CCAAT/enhancer binding protein) and E4BP4 (Adenovirus E4 promoter binding protein 4) were generated by site-directed mutagenesis and confirmed by DNA sequencing (Fig. 3.3). Plasmids expressing C/EBPα [206] and E4BP4 (Open Biosystems) were used.SREBP-1-targeting microRNA (miRNA) with target sequence of 5' CCTGGTCTACCATAAGCTGCA 3' was constructed in pcDNA6.2-GW/EmGFP miR vector (Invitrogen).

SREBP-1, Flag (M2), fibrillarin, β-actin and Myc epitope antibodies were from Santa Cruz Biotechnology, Sigma-Aldrich, and Cell Signaling Technology, respectively. Anti-HBx antibody was provided by Richardson [201].

3.5.2 Cell culture, transfection and nuclear fractionation

Huh-7 cells [207] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Huh-7 cells were transfected using the calcium phosphate precipitation method as previously described [163]. Nuclear fractions were isolated as described [167].

3.5.3 Immunoblotting analysis

Huh-7 cells were collected with a Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Immunoblotting was performed as described [164,167]. For SREBP experiments, cells were treated with a protease inhibitor ALLN (25 μg/ml, Calbiochem) for 1 h prior to lysis.

3.5.4 Reverse transcription and real-time PCR

RNA was isolated from Huh-7 cells with Trizol (Invitrogen) followed by DNase I (Invitrogen) digestion. Reverse transcription was carried out by Superscript II (Invitrogen) and random priming. Real-time PCR was performed with primers SREBP-1a-FD (5' CGCTGCTGACCGACAT 3') and SREBP-1a-rev (5' CAAGAGAGGAGCTCAATG 3') using SYBR Green based detection system. Housekeeping gene GUSB was amplified in parallel by primers GUSB-FD (5' GGTGCTGAGGATTGGCAGTG 3') and GUSB-rev (5' CGCACTTCCAACTTGAACAGG 3'). Data was analyzed by Bio-RadiQ5 program.

3.5.5 Luciferase assay

Huh-7 cells were lysed in a Passive Lysis Buffer (Promega) and luciferase activity was determined using luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Results were analyzed for statistical differences using Student's *t* test. A p value of ≤ 0.05 was considered statistically significant.

3.6 Results

3.6.1 HBx increases the level of SREBP-1a in the nucleus

HBx coding sequence was cloned into the pEF-cyto-myc vector in-frame with the myc tag. The resulting plasmid was transfected into Huh-7 cells and the cell lysates were analyzed in immunoblotting. As shown in Fig. 3.1A, a specific protein band was recognized by an HBx-specific antibody and a myc-tag antibody (not shown) in cells transfected with HBx-expressing plasmid, but not in vector transfected cells. The level of β-actin was used as loading control. These results demonstrated the expression of HBx protein after transfection.

Given the importance of SREBP-1a in modulating lipid metabolism, we explored the role of HBx in SREBP-1a activation. Huh-7 cells were transfected with HBx-expressing plasmid and vector control. The level of SREBP-1 was analyzed by immunoblotting using an anti-SREBP-1 antibody. As shown in the upper panel of Fig. 3.1A, expression of HBx was associated with increased level of SREBP-1 compared to control. Because we were interested in SREBP-1a levels especially in the nucleus as the active form, however the SREBP-1 antibody cannot distinguish SREBP-1a from another isoform SREBP-1c. Therefore we used a plasmid expressing Flag-tagged SREBP-1a and an anti-Flag antibody to examine nuclear SREBP-1a levels (lower panel of Fig. 3.1A). The results showed that the nuclear SREBP-1a protein level was increased in HBx transfected cells than in vector transfected cells. To determine whether the increased SREBP-1a correlates with its enhanced activity as a transcription factor, we used an SRE-luciferase reporter where the luciferase expression is directly controlled by three copies of SRE sequences (ATCACCCCAC, pSRE-Luc) [204]. This is because SREBP-1a activates transcription of its target genes by binding to the SRE sequence. As shown in Fig. 3.1B, HBx expression significantly increased SRE-driven luciferase activity by SREBP-1a compared to control. These results indicate that expression of HBx increases SREBP-1a level in the nucleus and its transcription factor activity.

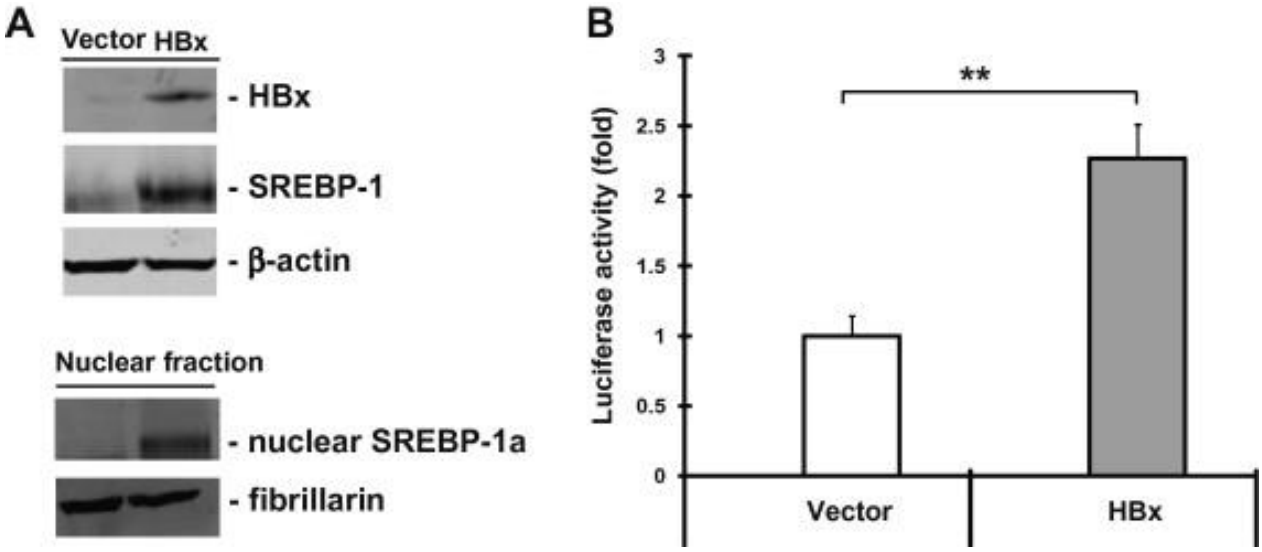

Fig. 3.1. Expression of HBx increases the level of nuclear SREBP-1a and its transactivation activity. (A) In the top panel, the levels of HBx, SREBP-1, and β-actin in Huh-7 cells after transfection with an HBx-expressing plasmid or the vector were analyzed by immunoblotting. In the bottom panel, levels of SREBP-1a in the nuclear fraction were analyzed by immunoblotting after co-transfection with plasmids expressing Flag-SREBP-1a and HBx or vector. The blots were probed with antibodies against Flag-tag or fibrillarin. This experiment was performed three times. (B) A luciferase reporter driven by SRE sequences was co-transfected with a plasmid encoding Flag-SREBP-1a together with HBx-expressing plasmid or vector control into Huh-7 cells. Luciferase assay was performed using the cell lysates. Luciferase activity was expressed as fold change relative to vector control. The statistical difference between samples was demonstrated as ** if $p \leq 0.01$. This experiment was performed three times.

3.6.2 HBx activates SREBP-1a transcription

SREBPs are often regulated at the transcription level. To determine whether HBx regulates SREBP-1a transcription, we measured the level of SREBP-1a transcript by real-time PCR after HBx expression in Huh-7 cells. As shown in Fig. 3.2A, HBx expression resulted in more than 2-fold increase in SREBP-1a transcript level in comparison to control. A greater than one genome length HBV plasmid is a widely used model for HBV. As such, we transfected HBV wild-type genome (pawy1.2) and HBV without HBx (pawy1.2*7) [200] into Huh-7 cells and determined SREBP-1a transcript levels. As shown in Fig. 3.2B, SREBP-1a transcript level in HBV-transfected cells was significantly higher than those in vector- or HBV ΔHBx-transfected cells. The expression of HBx was confirmed after HBV plasmid transfection, whereas no HBx could be detected in vector- or HBV ΔHBx-transfected cells (Fig. 3.2C). These results indicate that HBx up-regulates SREBP-1a transcription.

To further characterize SREBP-1a transcription up-regulation by HBx, Huh-7 cells were co-transfected with a luciferase reporter under the control of human SREBP-1a promoter (-1008) [205] and increasing amounts of HBx-expressing plasmid. The total amounts of plasmid DNA used for transfection were kept constant by adding appropriate amounts of the vector plasmid. Luciferase assay showed that HBx significantly activated SREBP-1a promoter activity in a dose-dependent manner in comparison to vector control (Fig. 3.2D). Similarly, HBV plasmid transfection resulted in significantly higher luciferase activity than vector- or HBV ΔHBx-transfection (Fig. 3.2E).

Next, we wanted to map the regions on the SREBP-1a promoter that were required for its activation by HBx. We used five truncatedSREBP-1a promoters -889, -717, -436, -398, and -360 (Fig. 3.3A). Luciferase assay results showed that deletion from -1008 to -436 in the SREBP-1a promoter did not affect its activation by HBx, whereas the activation was abolished when the -398 and -360 promoters were used. These results indicate that the sequence between -436 and -398 in the SREBP-1a promoter is required for its activation by HBx.

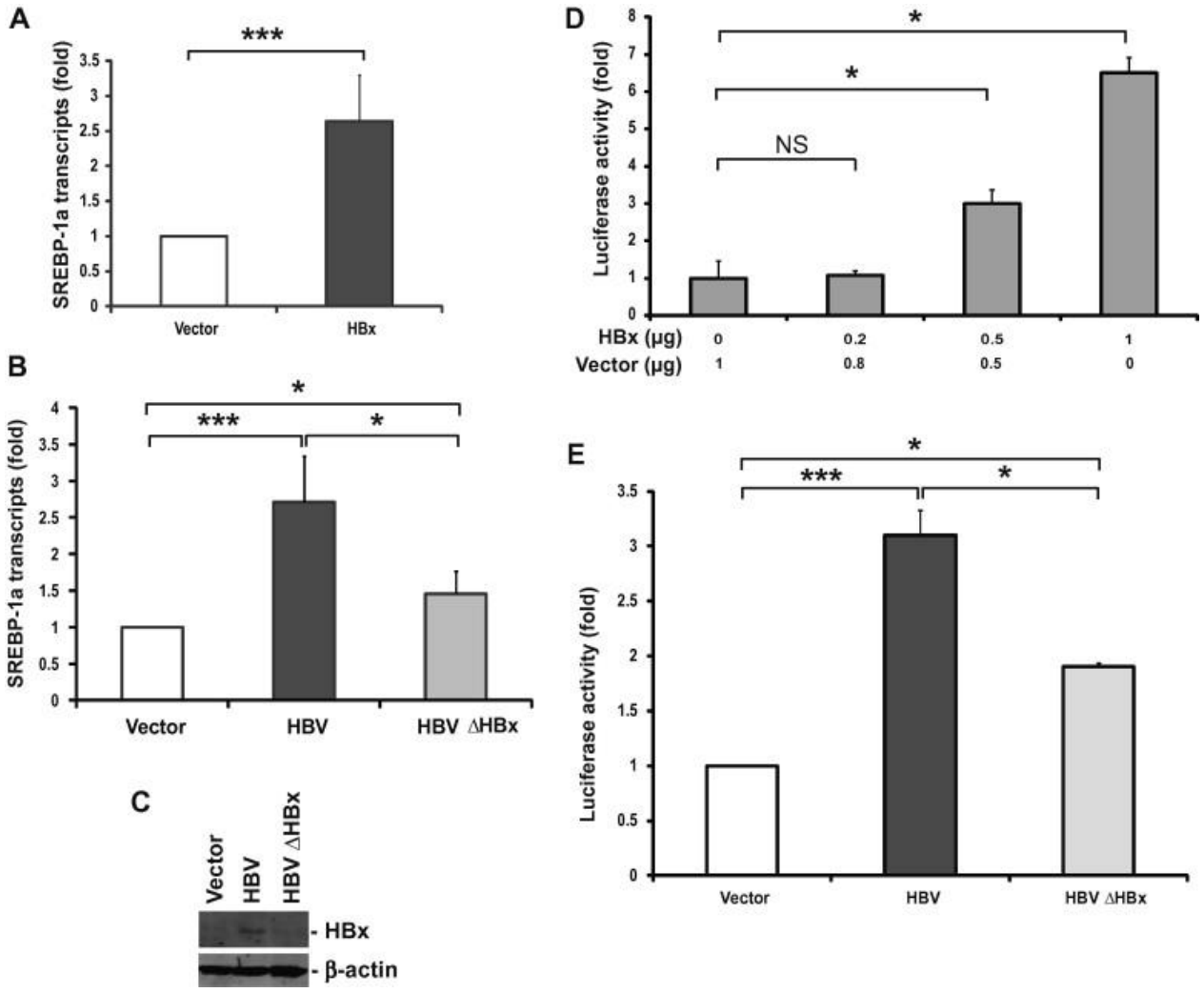

Fig. 3.2. HBx up-regulates SREBP-1a transcription. (A and B) Huh-7 cells were transfected with vector or HBx-expressing plasmids (A), vector, HBV, or HBV ΔHBx (B). The levels of SREBP-1a transcript were analyzed by reverse-transcription real-time PCR. These experiments were performed three times. (C) The protein levels of HBx and β-actin in Huh-7 cells after transfection with vector, HBV, or HBV ΔHBx were determined by immunoblotting. This experiment was performed three times. (D and E) Huh-7 cells were co-transfected with a human SREBP-1a promoter (-1008/+194)-luciferase reporter plasmid with increasing amounts of HBx-expressing plasmid (D) or vector, HBV, or HBV ΔHBx (E). Luciferase activities were expressed as fold changes relative to vector control. The statistical differences between samples were demonstrated as *NS* for not significant, * if $p \leq 0.05$, or *** if $p \leq 0.001$. These experiments were performed three times.

3.6.3 Effects of C/EBP and E4BP4 on SREBP-1a regulation by HBx

Sequence analysis by the MatINSPECTOR prediction program indicated that the -436 to -398 region in the SREBP-1a promoter contains binding motifs for transcription factors C/EBP and E4BP4 (Fig. 3.3B). To gain the first insights into the role of these two factors in SREBP-1a regulation by HBx, we mutated the binding sequences for each of the factors in the SREBP-1a promoter (Fig. 3.3C). These mutant SREBP-1a promoters were used in co-transfection experiments to test their activity after HBx expression. As shown in Fig. 3.3C, HBx failed to activate these mutant SREBP-1a promoters. These results suggested that the integrity of C/EBP and E4BP4 binding sequences is necessary for SREBP-1a regulation by HBx. Previous research has established that C/EBP and E4BP4 have divergent effects on transcription, although both belong to a family of basic leucine zipper (bZIP) proteins [208,209].

To determine the effects of these transcription factors in SREBP-1a regulation by HBx, we studied SREBP-1a promoter activity after ectopic expression of C/EBPα or E4BP4. There are six isoforms in the C/EBP family and C/EBPα was isolated from the liver [31,34].

Therefore, we used a plasmid expressing C/EBPα. As shown in Fig. 3.3D, transfection with increasing amounts of C/EBPα resulted in dose-dependent SREBP-1a promoter activation in both vector and HBx expressing cells. In contrast, increasing amounts of E4BP4 resulted in dose-dependent decrease in SREBP-1a promoter activity in both vector and HBx expressing cells (Fig. 3.3E). These results indicate that C/EBPα is as an activator, whereas E4BP4 is a repressor for SREBP-1a promoter regulation by HBx.

Opposing effects of C/EBP and E4BP4 on SREBP-1a promoter regulation by HBx raised a question as to whether the inhibitory effect of E4BP4 can be overcome by C/EBP. To answer this question, Huh-7 cells were co-transfected with HBx-expressing plasmid and SREBP-1a promoter (-436) - luciferase reporter, together with E4BP4 alone or with C/EBPα. Corresponding vectors were used as controls. As shown in Fig. 3.3F, the inhibition of SREBP-1a promoter activity by E4BP4 was significantly reversed upon ectopic expression of C/EBPα. These results suggest that C/EBPα has a dominantly activating effect on SREBP-1a promoter activity.

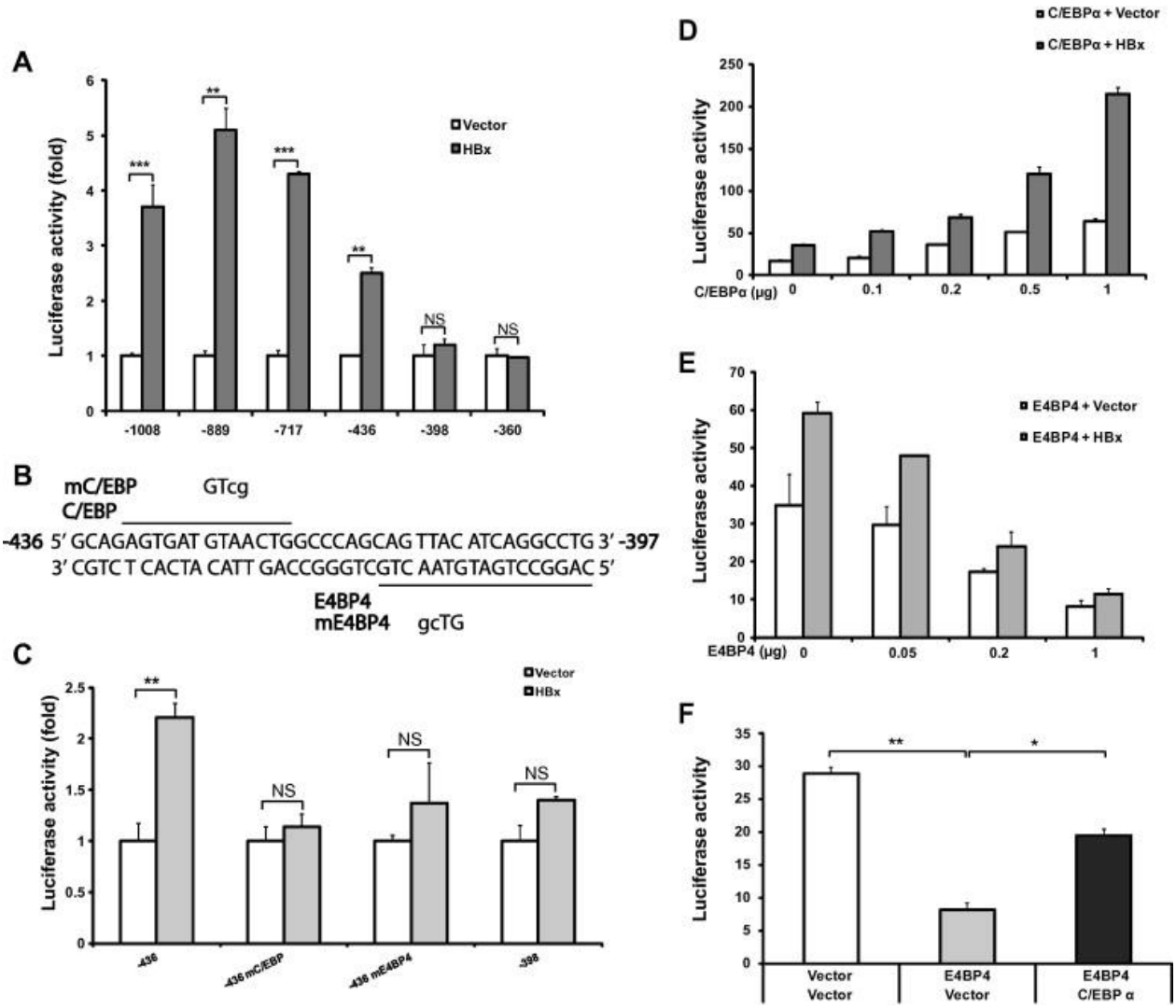

Fig. 3.3. The roles of transcription factors C/EBP and E4BP4 in SREBP-1a promoter regulation by HBx. (A) Huh-7 cells were transfected with SREBP-1a promoters of different lengths together with HBx-expressing plasmid or vector control. SREBP-1a promoter activity was determined by luciferase assay. (B) SREBP-1a promoter sequence -436 to -397. The binding motifs as well as the mutated sequences for C/EBP and E4BP4 are shown. (C) Sequence integrity of the SREBP-1a promoter -436 to -398 region is required for its activation by HBx. Huh-7 cells were co-transfected with wild-type or mutant SREBP-1a promoters with HBx-expressing plasmid or vector control. SREBP-1a promoter activity was determined by luciferase assay. (D and E) Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (-436/+194), HBx-expressing plasmid or vector control, together with increasing amounts of C/EBPα (D) or E4BP4 (E). SREBP-1a promoter activity was determined by luciferase assay. (F) Huh-7 cells were co-transfected with SREBP-1a-promoter luciferase reporter (-436/+194), HBx-expressing plasmid, together with vector, E4BP4-expressing plasmid, and C/EBPα-expressing plasmid. SREBP-1a promoter activity was determined by luciferase assay. The statistical differences between samples were demonstrated as *NS* for not significant, * if $p \leq 0.05$, ** if $p \leq 0.01$, or *** if $p \leq 0.001$. All experiments were performed three times.

3.6.4 Effect of SREBP-1 in HBV enhancer II/core promoter activation by HBx

Our results so far have shown that HBx activates SREBP-1a. Previous studies demonstrated that HBx increases HBV enhancer II/core promoter activity [11,196]. We therefore were interested in determining whether SREBP-1a is involved in this process. For this purpose, we used a miRNA to knock down SREBP-1 expression in Huh-7 cells. As shown in Fig. 3.4A, the levels of both precursor and mature SREBP-1 proteins were reduced by SREBP-1 miRNA in comparison to control miRNA. When Huh-7 cells were transfected with vector or HBx-expressing plasmids together with pGL4-HBV EN2/CP, modest but significant 1.5-fold transactivation of enhancer II/core promoter by HBx was observed (Fig. 3.4B). This result is consistent with other studies showing activation of enhancer II/core promoter by HBx [11,196]. Upon knocking down SREBP-1, HBx was no longer able to transactivate enhancer II/core promoter (Fig. 3.4C). These results demonstrate that SREBP-1 is involved in HBV enhancer II/core promoter transactivation by HBx.

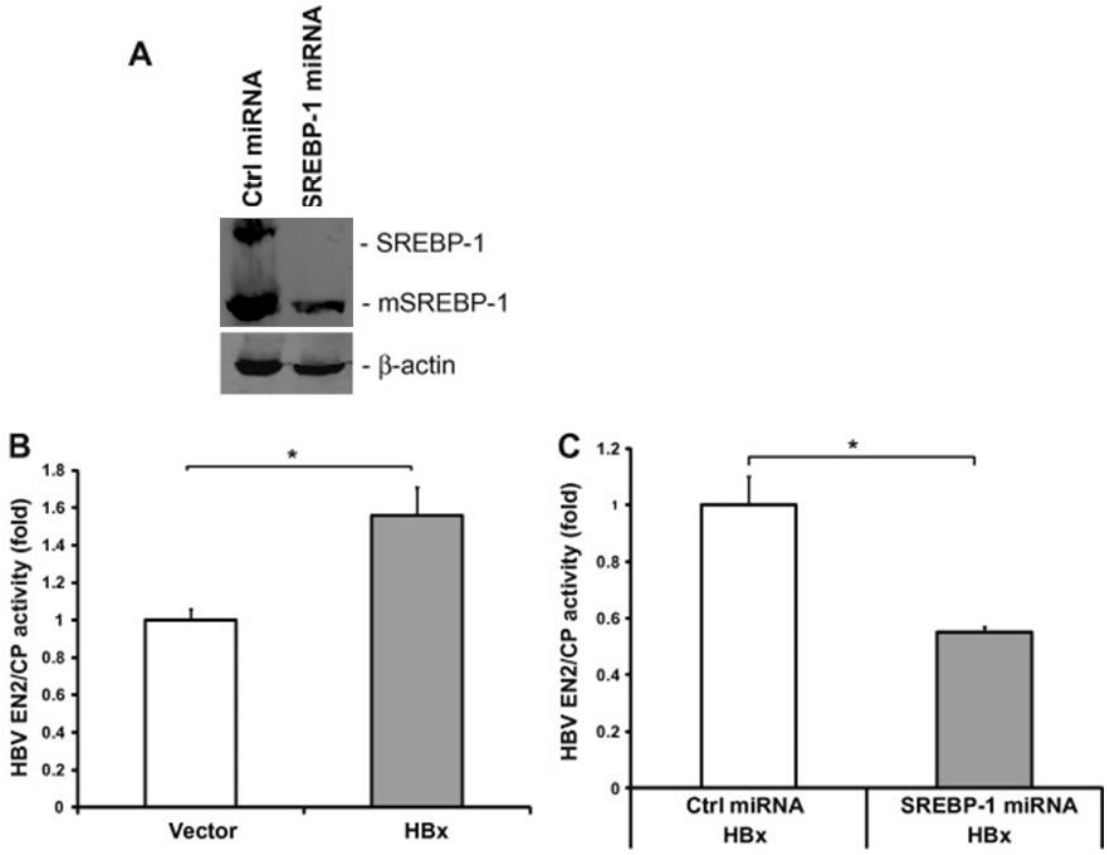

Fig. 3.4. The role of SREBP-1 in HBV enhancer II/core promoter activation by HBx. (A) Huh-7 cells were transfected with control or SREBP-1 miRNAs. The levels of precursor and mature SREBP-1 were determined by immunoblotting. This experiment was performed three times. (B) Huh-7 cells were co-transfected with HBV enhancer II/core promoter-luciferase reporter pGL4 HBV EN2/CP and vector or HBx-expressing plasmids. Luciferase activities after HBx expression were expressed as fold changes relative to vector control. (C) Huh-7 cells were co-transfected with pGL4 HBV EN2/CP, HBx-expressing plasmid, and control or SREBP-1 miRNA. Luciferase activities after SREBP-1 knockdown were expressed as fold changes relative to control miRNA-transfection. The statistical differences between samples were demonstrated as * if $p \leq 0.05$. These experiments were performed three times.

3.7 Discussion

HBx is a multifunctional protein. Here, we demonstrated that HBx activates SREBP-1a, a transcription factor involved in several (patho)-physiological conditions such as lipogenesis and carcinogenesis [176,210]. We further demonstrated that SREBP-1 is involved in HBV enhancer II/core promoter activation by HBx. To become an active transcription factor, SREBP-1a needs to enter the nucleus and therefore nuclear SREBP-1a level reflects its activity. As such, we first demonstrated that HBx expression is associated with increased level of SREBP-1a in the nucleus (Fig. 3.1A). An SRE-driven luciferase reporter assay confirmed that SREBP-1a in HBx-transfected cells is indeed transcriptionally active (Fig. 3.1B).

The expression of SREBP-1a can be regulated at the transcription level [205]. Our results demonstrated that HBx can significantly up-regulate the activity of SREBP-1a promoter in a dose-dependent manner (Fig. 3.2D). More importantly, we showed that HBx in the context of HBV can also transactivate SREBP-1a transcription using a plasmid-based HBV model (Fig. 3.2B and E). Mapping the sequences in the SREBP-1a promoter required for activation by HBx led us to concentrate on two bZIP transcription factors, namely C/EBP and E4BP4. Mutating the C/EBP binding sequence on the SREBP-1a promoter canceled SREBP-1a promoter activation by HBx (Fig. 3.3C). Consistently, ectopic expression of C/EBPα demonstrated that it can further enhance SREBP-1a promoter activation by HBx (Fig. 3.3D). These results suggest that C/EBP is required for SREBP-1a activation by HBx.

Ectopic expression of E4BP4, on the other hand, has an opposing, inhibiting effect on SREBP-1a promoter activity (Fig. 3.3E). This finding is consistent with the established repressor activity of E4BP4 on transcription [208]. However, when the E4BP4 binding sequence was mutated on the SREBP-1a promoter, we did not see the expected increase of the promoter activity (Fig. 3.3C). The underlying reason is not clear. Several possible mechanisms exist. The binding sequences on gene promoters for bZIP transcription factors are similar, which may result in DNA binding competition by these factors [208]. In fact, it has been shown that C/EBP and E4BP4 can compete with each other in DNA binding and regulate transcription in a competitive manner [211,212]. Therefore, we cannot exclude the possibility that C/EBP can also bind the E4BP4 sequence on SREBP-1a promoter and positively regulate its activity. As such, elimination of E4BP4 binding sequence can potentially affect the activities of both E4BP4 and C/EBP.

Another possibility is that mutating the E4BP4 binding motif might somehow negatively affect the binding of C/EBP to its recognition sequence on the SREBP-1a promoter because they are in a very close proximity. In addition, b-ZIP factors regulate transcription by forming homo- and/or hetero-dimers [213]. According to a molecular interaction model, C/EBP and E4BP4 may interact with each other due to the presence of an asparagine residue in the "a" position of their bZIP domains which would favor heterodimerization [214]. It is reasonable to assume that elimination of E4BP4 binding sequence on the SREBP-1a promoter may increase the amount of free E4BP4. Then it is possible that more E4BP4 proteins can interact with C/EBP and interfere with the activity of C/EBP, resulting in reduced SREBP-1a promoter activation. If this is true, one would expect that increasing the amount of C/EBP should increase SREBP-1a promoter activity in the presence of E4BP4. This has been actually observed in our experiments (Fig. 3.3F). However, the exact mechanisms warrant further investigation.

Recent studies have shown that HBx can activate SREBP-1c, the other isoform of SREBP-1, through liver X receptor [109,110,215]. Adding to these previous findings, our study has demonstrated that HBx can also activate SREBP-1a through a different and complex mechanism involving at least two transcription factors, C/EBP and E4BP4. More importantly, SREBP-1a is a more potent transcription activator for both fatty acid and cholesterol synthesis pathways [68-70]. In contrast, SREBP-1c is less active and only activates fatty acid synthesis. The functional significance of SREBP-1 activation in HBV biology and pathogenesis has not been characterized. Towards this goal, we showed that knocking down SREBP-1 abolishes activation of HBV enhancer II/core promoter activation by HBx (Fig. 3.4). Since the miRNA sequence we used does not distinguish between SREBP-1a and -1c, further experiments are needed to determine isoform-specific effects of SREBP-1.

In conclusion, our results demonstrated that HBx activates the SREBP-1a activity by a complex mechanism involving two bZIP transcription factors C/EBP and E4BP4 with the former being the dominant factor leading to SREBP-1a promoter activation. Furthermore, we showed that SREBP-1 is involved in HBV enhancer II/core promoter activation by HBx.

3.8 Acknowledgments

We thank Drs. Betty Slagle, Christopher Richardson, Hitoshi Shimano, Ralf Bartenschlager, Margarita Melegari, and Ormond A. MacDougald for sharing reagents. This work was supported by Canadian Institutes of Health Research (CIHR) (ROP-88064 and RSN-109427) and Saskatchewan Health Research Foundation (SHRF). X.L. is a recipient of a post-doctoral fellowship from SHRF. This paper is VIDO Manuscript #623.

4.0 THE EFFECT OF HBX LOCALIZATION ON THE REGULATION OF SREBP-1 ACTIVITY

In the previous chapter, we demonstrated that HBx increases mature SREBP-1a protein level in the nucleus and its activity as a transcription factor. Moreover, we observed that this process is involved in up-regulation of HBV enhancer II/core promoter activity. Therefore the up-regulation of SREBP-1a activity by HBx may be involved in the context of HBV replication. HBx contains 154 amino acids and is localized into the nucleus, cytoplasm, and mitochondria. Different subcellular localizations of HBx display different transactivational activities. SREBP-1a is a transcription factor, the mechanisms of how HBx regulates SREBP-1a is still not fully understood. In order to understand the role of HBx in the regulation of SREBP-1a and HBV replication, in the following chapter we set to determine if different subcellular localizations of HBx differentially regulate SREBP-1a and HBV enhancers.

5.0 STRONGER ACTIVATION OF SREBP-1A BY NUCLEUS LOCALIZED HBX

Qi Wu [a], Ling Qiao [b, 1], Jian Yang [c], Yan Zhou [d], Qiang Liu [d, *]

[a] VIDO-InterVac, Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada

[b] VIDO-InterVac, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[c] Drug Discovery Group, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[d] VIDO-InterVac, Veterinary Microbiology, Vaccinology and Immunotherapeutics, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Keywords: HBx; SREBP-1a; Fatty acid synthase; Lipid accumulation; cell proliferation; HBV enhancers/promoters and replication

* Corresponding author.

VIDO-InterVac

University of Saskatchewan,

120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.

Fax: +1 306 966 7478.

E-mail address: qiang.liu@usask.ca. (Q. Liu).

[1] Current address: 2011 Collaborative Innovation Center of Tianjin for Medical Epigenetics, Key Laboratory of Hormones and Development (Ministry of Health), Metabolic Diseases Hospital & Tianjin Institute of Endocrinology, Tianjin Medical University, Tianjin, China.

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5.2 Authors' contribution

All the experiments within this chapter were performed by Qi Wu. The manuscript was written by Qi Wu and edited by Qiang Liu.

5.3 Abstract

We previously showed that hepatitis B virus (HBV) X protein activates the sterol regulatory elementbindingprotein-1a (SREBP-1a). Here we examined the role of nuclear localization of HBx in this process. In comparison to the wild-type and cytoplasmic HBx, nuclear HBx had stronger effects on SREBP-1a and fatty acid synthase transcription activation, intracellular lipid accumulation and cell proliferation. Furthermore, nuclear HBx could activate HBV enhancer I/X promoter and was more effective on up-regulating HBV mRNA level in the context of HBV replication than the wild-type HBx, while the cytoplasmic HBx had no effect. Our results demonstrate the functional significance of the nucleus-localized HBx in regulating host lipogenic pathway and HBV replication.

5.4 Introduction

Hepatitis B virus (HBV) is a partially double-stranded circular DNA virus belongs to the Hepadnavirus family [216]. HBV infection causes acute and chronic liver disease and is a high risk factor for the development of hepatocellular carcinoma (HCC) [217]. HBV X protein (HBx) functions as a transcriptional transactivator of host genes and promotes cell growth and possibly HCC [18,100]. HBV gene expression is regulated by two HBV promoters/enhancers [218]. HBx activates both elements and thus increases HBV replication [11,12]. HBx localizes in the nucleus and the cytoplasm, which has functional implications [123-125]. Different subcellular localizations of HBx display different transactivation activities. In comparison to the wild-type HBx, nucleus-localized HBx significantly activates HBV enhancer I/X but not NF-κB or AP-1; on the other hand, cytoplasmic HBx is crucial for activating the Ras-Raf-MAP kinase, NF-κB and AP-1 [126,127]. Nuclear, but not cytoplasmic, HBx is required for HBV replication in cell culture [129].

Sterol regulatory element-binding protein-1a (SREBP-1a) is a potent transcription factor for genes involved in fatty acid synthesis and cholesterol synthesis [69,70]. Our previous study showed that HBx activates SREBP-1a at the transcription level involving two transcriptional factors C/EBP and E4BP4 [137]. However, whether the subcellular localization of HBx plays a role in this process has not been investigated. In this study, we demonstrated that, in comparison to the cytoplasmic HBx, the nucleus-localized HBx has stronger effects on up-regulating SREBP-1a, fatty acid synthase (FASN), lipid accumulation, cell proliferation, HBV enhancer I/X promoter, and HBV mRNA in the context of HBV replication.

5.5 Materials and Methods

5.5.1 Plasmids

A plasmid expressing HBx with a myc-tag at the C-terminus under the control of the elongation factor-1a promoter, HBV enhancer II/core promoter luciferase reporter, SREBP-1a and FASN promoter luciferase reporters, and a greater-than-unit-length HBV genome without expressing HBx (payw 1.2*7) were described previously [137,163,219]. A nuclear localization signal (NLS, PKKKRKVFL) [126] or a nuclear export signal (NES, LALKLAGLDI) [129] was added to the N-terminus of the HBx coding sequence to create NLS-HBx or NES-HBx, respectively. The HBV enhancer I/X promoter sequence [220] was cloned upstream of the luciferase gene into the pGL4.14 vector (Promega), generating the pGL4-HBV enhancer I/X promoter.

5.5.2 Cell culture, transfection, and nuclear fractionation

Huh-7 cells [207] were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Transfection and nuclear fractionation were described previously [163,167].

5.5.3 Western blotting and antibodies

Western blotting was performed as described [164,167]. Fibrillarin, β-actin and myc epitope antibodies were from Sigma-Aldrich and Cell Signaling Technology, respectively.

5.5.4 Reverse transcription and real-time PCR

RNA extraction, reverse transcription, and real-time PCR were performed as described [137,221]. The primers for the real-time PCR were SREBP-1a-FD (5' CGCTGCTGACCGACAT

3') and SREBP-1a-rev (5' CAAGAGAGGAGCTCAATG 3'), FASN-FD (5' TCATCCCCCTGATGAAGAAG 3') and FASN-rev (5' ACTCCACAGGTGGGAACAAG 3'), HBV-FD (5' AGAAACAACACATAGCGCCTCAT 3') and HBV-rev (5' TGCCCCATGCTGTAGATCTTG 3'), and β-glucuronidase (GUSB)-FD (5' GGTGCTGAGGATTGGCAGTG 3') and GUSB-rev (5' CGCACTTCCAACTTGAACAGG 3').

5.5.5 Luciferase assay

Cells were lysed in a Passive Lysis Buffer (Promega) and the luciferase activity was determined using the luciferase assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs).After normalization against the total protein concentration in the same sample, the luciferase results were analyzed for statistical differences using Student's *t* test. A p value of ≤ 0.05 was considered statistically significant.

5.5.6 Oil Red O staining and MTT assay

The amounts of the neutral lipids were measured at 500 nm [168] after Oil Red O (ORO) staining as previously described [163]. The MTT assay was performed as described [221].

5.6 Results and Discussion

5.6.1 HBx subcellular localization analyzed by nuclear fractionation

Previous studies have shown that HBx in different subcellular compartments may have different functions [126,127,129]. However, the impact of the subcellular localization of HBx on lipogenic gene expression has not been studied. To specifically study the function of nucleus- and cytoplasm-localized HBx, a nuclear localization signal (NLS, PKKKRKVFL; NLS-HBx) [126] or a nuclear export signal (NES, LALKLAGLDI; NES-HBx) [129] was added to the N-terminus of the HBx coding sequence [137]. This is a widely used experimental approach [126,129,222,223]. To determine the intracellular distribution of the HBx proteins, nuclear and cytoplasmic fractions as well as total cell lysates were prepared and subjected to Western blotting after transfecting Huh-7 cells with the HBx-expressing plasmids. As shown in Fig. 5.1, wild-type HBx was present in both nuclear and cytoplasmic fractions as expected; NES-HBx was found predominantly in the cytoplasmic fraction, whereas NLS-HBx was predominantly nuclear. These results demonstrated that addition of a nuclear export signal to HBx efficiently excludes HBx from the nucleus and a nuclear localization signal renders nuclear localization of HBx.

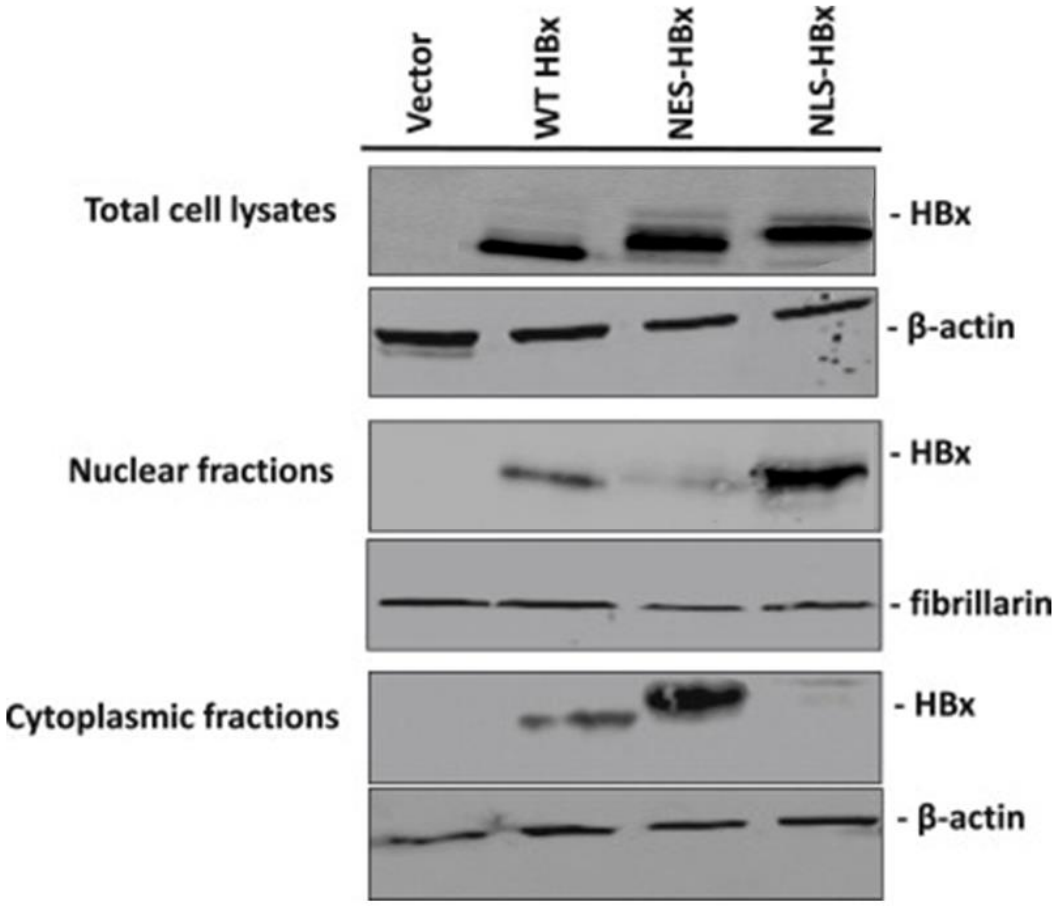

Fig. 5.1. Expression and subcellular localization of HBx proteins. Huh-7 cells were transfected with plasmids expressing wild-type (WT) HBx, HBx with a nuclear export signal (NES-HBx) or a nuclear localization signal (NLS-HBx) at the N-terminus, or vector. A myc tag is present at the C-terminus of HBx. At 48 h after transfection, total cell lysates, nuclear and cytoplasmic fractions were analyzed by Western blotting using antibodies against the myc tag, β-actin or fibrillarin. This experiment was performed five times.

5.6.2 Nuclear HBx is more effective on up-regulating SREBP-1a and FASN

We previously demonstrated that HBx activates SREBP-1a transcription [137]. To examine the role of subcellular localization of HBx in this process, we co-transfected Huh-7 cells with plasmids expressing wild-type HBx, NES-HBx, NLS-HBx, or empty vector, together with an SREBP-1a promoter luciferase reporter [137]. Consistent with our previous study [137], we observed significant activation of SREBP-1a promoter by wild-type HBx (Fig. 5.2A). Addition of a nuclear localization signal to HBx resulted in significantly higher SREBP-1a promoter activation than the wild-type HBx (Fig. 5.2A). In contrast, HBx with a nuclear export signal, NES-HBx, did not activate the SREBP-1a promoter (Fig. 5.2A). Since FASN is one of the target genes of SREBP-1a, we examined the effects of HBx subcellular localization mutants on FASN promoter activity using a FASN promoter luciferase reporter [163]. As shown in Fig. 5.2C, wild-type HBx resulted in significantly higher FASN promoter activity in comparison to vector. Addition of an NLS further enhanced FASN promoter activation by HBx, whereas the NES-HBx could no longer activate the FASN promoter (Fig. 5.2C). To substantiate the promoter-reporter results, we determined the transcript levels of endogenous SREBP-1a and FASN. As shown in Fig. 5.2B and D, the effects of HBx in different subcellular compartments on endogenous SREBP-1a and FASN transcript levels were in agreement with those obtained from promoter-reporter based assays (Fig. 5.2A and C). These results indicated that nucleus-localized HBx is more effective on the activation of SREBP-1a and FASN transcription. Since we previously showed that HBx activates SREBP-1a through transcriptional factor C/EBP [137] and others showed that HBx can enhance C/EBP activity through direct protein-protein interaction presumably in the nucleus [11], it is conceivable that the nuclear HBx is more readily for interacting with and activating C/EBP, which in turn increases SREBP-1a transcription.

5.6.3 Nuclear HBx is more effective on enhancing intracellular lipid accumulation and cell proliferation

To determine the effects of HBx subcellular localization on intracellular lipid accumulation, we measured the amounts of neutral lipids. As expected, wild-type HBx expression was associated with significantly more neutral lipids than vector (Fig. 5.2E). Lipid

accumulation was further enhanced by the NLS-HBx or returned to baseline by the NES-HBx (Fig. 5.2E). Since increased lipogenesis and lipid accumulation have been recognized as one of the major factors driving cell proliferation [224], we also determined the effects of HBx and its mutants on cell proliferation by a standard MTT assay. As shown in Fig. 5.2F, cell proliferation was significantly increased by wild-type HBx in comparison to vector control. Once again, this effect was enhanced by a nuclear localization signal and dampened by a nuclear export signal (Fig. 5.2F). These results indicated that nucleus-localized HBx is more effective on stimulating lipid accumulation and cell proliferation, probably as a consequence of enhanced SREBP-1a and FASN activity.

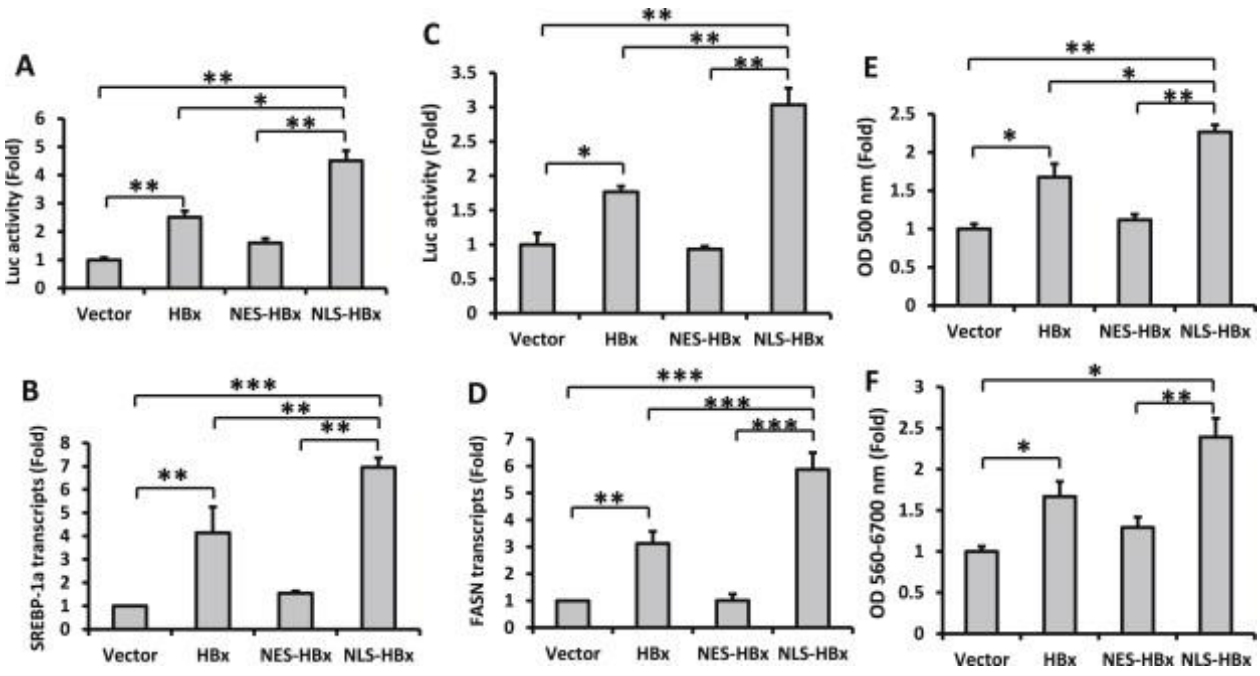

Fig. 5.2. Nuclear HBx has a stronger effect on SREBP-1a and FASN transcription activation, intracellular lipid accumulation and cell proliferation than wild-type and cytoplasmic HBx. (A and C). Huh-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with an SREBP-1a promoter (A) or a FASN promoter (C) luciferase reporters. Luciferase assay was performed at 48 h after transfection and normalized against the protein concentration to determine the promoter activities. (B and D). Huh-7 cells were transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector. At 16 h after transfection, the transcript levels of SREBP-1a (B) and FASN (D) were determined by reverse transcription real-time PCR. The levels of β-glucuronidase (GUSB) were also determined and used for normalization. (E and F). Huh-7 cells were transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector. At 48 h after transfection, cells were subjected to either Oil Red O staining (E) or MTT assay (F). Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, or ** if $p \leq 0.01$. All experiments were performed three times.

5.6.4 Nuclear HBx is more effective on up-regulating HBV mRNA

HBx also activates HBV enhancers and thus increases HBV replication [11,12]. To examine how HBx in different subcellular compartments regulates HBV enhancers, we co-transfected Huh-7 cells with plasmids expressing HBx, NES-HBx, NLS-HBx, or empty vector, and luciferase reporters under the control of HBV enhancer I/X [11] or HBV enhancer II/CP [123,137]. As expected, we observed more than 1.5-fold activation of HBV enhancers I and II by the wild-type HBx (Fig. 5.3A and B). NES-HBx did not activate either enhancers (Fig. 5.3A and B). NLS-HBx increased HBV enhancer I activity more than 2-fold than baseline (Fig. 5.3A); although higher than the wild-type HBx, it just fell short of reaching statistical significance (p = 0.052). NLS-HBx did not significantly increase HBV enhancer II/CP activity than vector or wild-type HBx (Fig. 5.3B). These results indicated that while the cytoplasmic HBx does not activate HBV enhancers/promoters, nuclear HBx can increase HBV enhancer I/X activity, but has a marginal effect on HBV enhancer II/CP activity. It has been shown that nuclear HBx can no longer activate NF-κB [126]. Interestingly, HBV enhancer II has an NF-κB binding motif whereas HBV enhance I does not [225]. Therefore, it is possible that the differential effects of nuclear HBx on two HBV enhancers/promoters are due to its ability to activate NF-κB activity. However, the exact mechanisms warrant further investigation. It is worth mentioning that only nuclear, but not cytoplasmic, HBx can rescue HBx-deficient virus replication [129]. The demonstration of HBV enhancer I/X activation by NLS-HBx by our results may have provided a possible mechanistic explanation.

To further understand the biological significance of the results we have so far, we determined the effect of HBx in different subcellular compartments on HBV mRNA levels in the context of HBV replication in a plasmid-based HBV replication system using a greater-than-unit-length HBV genome plasmid. We used an HBV genome without expressing HBx, payw 1.2*7 [219], which is a common approach to study the role of HBx in HBV replication [129,196]. Huh-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with HBV genome plasmid payw 1.2*7. At 48 h after transfection, the level of HBVmRNA was determined. As shown in Fig. 5.3C, wild-type HBx significantly increased HBV mRNA level by more than two folds in comparison to vector. This is consistent with the enhancing role of HBx in HBV replication [226]. While cytoplasmic HBx had no effect on HBV mRNA level, nuclear

HBx increased HBV mRNA level by six folds, which was significantly higher than wild-type HBx (Fig. 5.3C). These results demonstrated that the nuclear HBx is more effective on enhancing HBV mRNA levels in the context of HBV replication than the wild-type HBx. While our results are in agreement with those from the Slagle's group [129], they are not consistent with the results from the Ryu's group [223]. The reason for this discrepancy is not clear, but it may be due to different cell lines and/or the assays used. This has been thoroughly discussed recently [226].

Although HBV infection and HBx expression are risk factors for developing hepatocellular carcinoma, the molecular mechanisms are not well understood. A hallmark of carcinogenesis is the abnormally high demand for lipid synthesis by cancer cells. Therefore, activation of SREBP-mediated lipogenic pathways have been recognized as a critical oncogenic mechanism [227]. Clinical studies have found HBx mutations conferring nuclear localization in cancerous tissues at a high frequency [228,229]. Our study demonstrated a stronger activation of the SREBP-1a mediated lipogenic pathway by the nuclear HBx. As such, our results may help understand the biological significance of these clinical findings.

In summary, we have investigated the role of nuclear and cytoplasmic localization of HBx in modulating host lipogenic expression, lipid accumulation, cell proliferation, HBV regulatory elements, and HBV mRNA levels. Our results indicated that nucleus-localized HBx is more effective on activating SREBP-1a and FASN transcription, increasing intracellular lipid accumulation, and cell proliferation. While cytoplasm-localized HBx has no effect on HBV enhancer/promoter activities, nuclear localization plays an important role in enhancing HBV enhancer I/X promoter activity and HBV mRNA level by HBx. Our work sheds more light on the contribution of HBx to the pathogenesis and oncogenesis associated with HBV infection.

5.7 Acknowledgments

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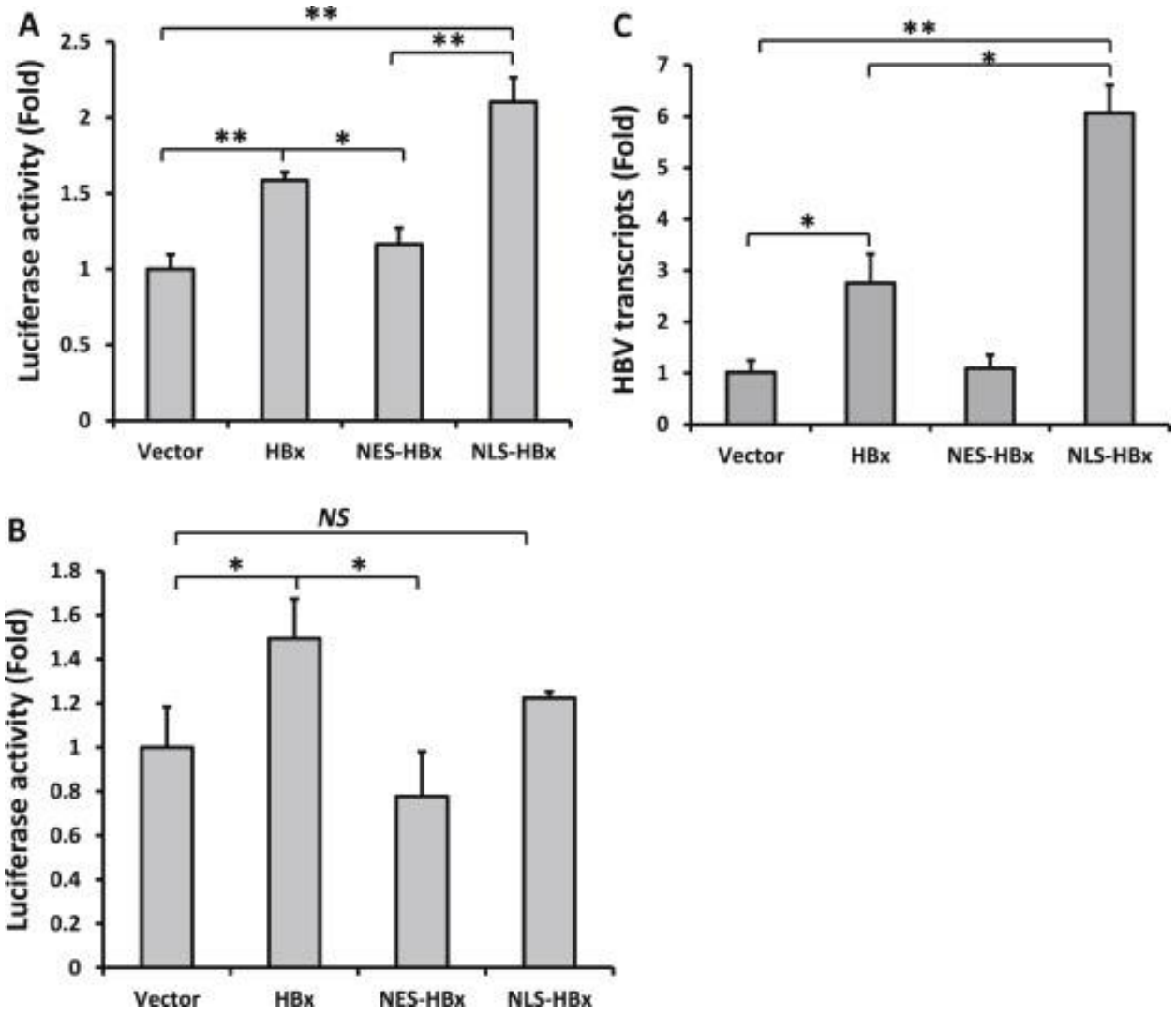

Fig. 5.3. The effect of HBx on HBV enhancer/promoter and HBV mRNA levels. Huh-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS-HBx, or vector, together with luciferase reporters under the control of HBV enhancer I/X promoter (A) or HBV enhancer II/core promoter (B). Luciferase assay was performed at 48 h after transfection and normalized against the protein concentration to determine the HBV enhancer/promoter activities. (C). Huh-7 cells were co-transfected with plasmids expressing WT, NES-, or NLS- HBx, or vector, together with a greater-than-unit-length HBV genome plasmid payw 1.2*7 (no HBx). At 48 h after transfection, the level of HBV mRNA was determined by reverse transcription real-time PCR. The level of β-glucuronidase (GUSB) was used for normalization. Statistical differences between samples, analyzed by Student's *t* test, were demonstrated as * if $p \leq 0.05$, or ** if $p \leq 0.01$. All experiments were performed three times.

6.0 DOMAIN OF HBX THAT REGULATES SREBP-1A AND HBV REPLICATION

In Chapter 3, we attempted to determine that HBx acts as a transcription factor which enhances the mature SREBP-1a protein level. In Chapter 5, we further demonstrated that nucleus-localized HBx, in comparison to the wild-type and cytoplasm-localized HBx, has stronger effects on SREBP-1a and FASN transcription activation, intracellular lipid accumulation and cell proliferation. HBx is a small protein consisting of 154 amino acids. Truncated forms of HBx are often found in the tissues after HBV infection. Truncated forms of HBx play different roles in HBx stability and functions. In the following chapter we set to determine if different truncated forms of HBx differentially regulate SREBP-1a and HBV replication.

7.0 HBX TRUNCATION MUTANTS DIFFERENTIALLY MODULATE SREBP-1A AND -1C TRANSCRIPTION AND HBV REPLICATION

Qi Wu [a], Qiang Liu [b, *]

[a] VIDO-InterVac, Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada

[b] VIDO-InterVac, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Keywords: HBx truncations; SREBP-1a and -1c; Fatty acid synthase; HBV enhancers; HBV transcription; HBV replication

* Corresponding author.

VIDO-InterVac

University of Saskatchewan,

120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.

Fax: +1 306 966 7478.

E-mail address: qiang.liu@usask.ca. (Q. Liu).

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7.2 Authors' contribution

All the experiments within this chapter were performed by Qi Wu. The manuscript was written by Qi Wu and edited by Qiang Liu.

7.3 Abstract

As master transcription factors for lipogenesis, sterol regulatory element-binding protein-1 (SREBP-1) has two isoforms, SREBP-1a and SREBP-1c. Hepatitis B virus X (HBx) can up-regulate the transcription of both SREBP-1a and SREBP-1c. HBx is a small protein consisting of 154 amino acids. Truncated forms of HBx, often found in the tissues after HBV infection, may have a role in the pathogenesis associated with HBV infection. In this study, we examined the effects of two HBx truncation mutants, HBx aa. 1-127 and HBx aa. 43-154, on the transcription of SREBP-1a and SREBP-1c. HBx 1-127 can up-regulate SREBP-1c, but not SREBP-1a transcription, whereas HBx 43-154 can activate SREBP-1a, but not SREBP-1c transcription. We further determined the activities of two HBV enhancers after the expression of the truncated HBx proteins. HBx 1-127 and HBx 43-154 can only up-regulate HBV enhancer I or HBV enhancer II, respectively. Knocking down SREBP-1 abrogates enhancer activation by HBx proteins, suggesting a role of SREBP-1. In addition, using HBV enhancer mutants, we found that the binding sequence for AP-1 on enhancer I is essential for its activation by HBx 1-127, whereas C/EBP and Sp1 sites are required for enhancer II activation by HBx 43-154. Finally, we showed

that both HBx 1-127 and HBx 43-154 can increase HBV transcription and HBV replication dependent upon SREBP-1 because knocking down SREBP-1 abrogates the up-regulation. Furthermore, upon ectopic expression of either SREBP-1a or SREBP-1c, we showed that SREBP-1a is involved in HBV transcription and replication up-regulation by HBx 43-154, whereas SREBP-1c is involved in HBV transcription and replication up-regulation by HBx 1-127. Our results should help understand the interactions between HBV and the SREBP-1-mediated lipogenic pathway.

7.4 Main Text

Sterol regulatory element-binding proteins (SREBPs) are key regulators of lipogenic gene transcription [67,230,231]. In humans, two SREBP genes encoding three proteins have been identified. SREBP-1a and -1c are translated from alternative transcripts encoded by a single gene, whereas SREBP-2 has its own gene. The newly synthesized precursor SREBPs are bound to the ER forming a complex with SREBP-cleavage activating protein (SCAP) [193]. Upon stimulation, the precursor SREBP proteins are transported from ER to the Golgi apparatus by SCAP [232].Once in the Golgi, the N-terminal domain of SREBP is released after proteolytic digestions and transported to the nucleus. The mature SREBP (mSREBP) proteins are active transcription factors. SREBP-1a functions as a potent activator of all SREBP-responsive genes involved in fatty acid synthesis and cholesterol synthesis, while SREBP-1c only activates fatty acid synthesis but not cholesterol synthesis [69].

Hepatitis B virus (HBV) X protein (HBx) is a multi-functional protein [225,226]. In comparison to non-tumor tissues, truncated forms of HBx are more commonly found in HBV-associated tumor tissues, presumably after the integration of HBV genomic DNA into the host genome [130-132]. The pathobiological significance of this finding is not well understood. Several studies have demonstrated that the C-terminal region of HBx is essential for HBx stability [132-134]. In addition, the C-terminal region of HBx plays important roles in stimulating HBV replication by HBx in cell culture [12,133,135]. Deletion of 23 amino acids at its C-terminal end of HBx abrogates its ability to activate NF-κB [133]. Amino acids 58-119 of HBx are required for activating the MAP kinase pathway [128].

HBV transcription is regulated by two viral enhancers and four promoters [225]. HBx protein has been shown to be able to activate both enhancers [11,12]. The regions of HBx for this function have not been mapped. From the pathobiological standpoint, the effects of truncated HBx proteins often expressed by the integrated HBV DNA on HBV enhancers are not well understood, although it has been suggested that HBV enhancers are kept intact upon integration [233]. As such, it is necessary to study the effects of truncated HBx on the activity of HBV enhancers.

Several studies including ours have reported that either full-length or truncated HBx proteins can activate the transcription of SREBP-1a and SREBP-1c [110,137,234]. To extend these findings, we demonstrated in this study that the N- and C-terminally truncated forms of HBx differentially modulate the transcription of SREBP-1a and SREBP-1c, the activities of two HBV enhancers, and HBV replication.

To determine how truncated forms of HBx regulate the transcription of SREBP-1a and SREBP-1c, we generated two truncated forms of HBx, aa. 1-127 and 43-154 with an N-terminal Flag-tag [135,235], using a plasmid expressing full-length HBx (aa. 1-154) [137]. Huh-7 and HepG2 cells were transfected with these plasmids using the calcium phosphate precipitation method [236]. The expression of HBx proteins was confirmed by Western blotting [163] with a Flag-specific antibody (Sigma-Aldrich) (Fig. 7.1A). As sample loading controls, the levels of β-actin were also determined by a β-actin-specific antibody (Cell Signaling Technologies) (Fig. 7.1A). IRDye 800 CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences) were used as secondary antibodies.

To study the effects of HBx truncations on SREBP-1a and SREBP-1c transcription, we determined the transcript levels of SREBP-1a and -1c by quantitative real-time PCR as described [137,221]. The primers for the real-time PCR were SREBP-1a-FD (5' CGCTGCTGACCGACAT 3'), SREBP-1c-FD (5' GCCATGGATTGCACTTT 3'), and SREBP-1a/1c-rev (5' CAAGAGAGGAGCTCAATG 3'). The level of a housekeeping gene β-glucuronidase (GUSB) amplified by GUSB-FD (5' GGTGCTGAGGATTGGCAGTG 3') and GUSB-rev (5' CGCACTTCCAACTTGAACAGG 3') was used for normalization. Results were analyzed for statistical differences by Student's *t* test. A p value of ≤ 0.05 was considered statistically significant. As shown in Fig. 7.1B and C, full-length HBx could up-regulate the transcript levels of both SREBP-1a and -1c, consistent with previously published results [110,137]. Deletion of 27

amino acids from the C-terminus, HBx 1-127, could no longer increase SREBP-1a transcription, but remained the ability to up-regulate SREBP-1c transcription (Fig. 7.1B and C). In contrast, deletion of the N-terminal 42 amino acid, HBx 43-154, demonstrated the opposite effects: it could up-regulate SREBP-1a transcription, but had no effect on SREBP-1c transcription (Fig. 7.1B and C). These data demonstrate that HBx N- and C-terminal truncations exhibit differential effects on the transcription of SREBP-1a and -1c.

SREBP-1a and -1c are two isoforms of SREBP-1 with overlapping and distinct functions. The activity of SREBP-1 proteins is conferred by the mature SREBP-1 (mSREBP-1) proteins in the nucleus. We therefore determined the levels of SREBP-1 and mSREBP-1 proteins after expressing HBx truncations. Because of the lack of isoform-specific antibodies, we could only determine the overall SREBP-1 levels. As shown in Fig. 7.2, both HBx 1-127 and 43-154 could increase the SREBP-1 and mSREBP-1 levels in total cell lysates in comparison to vector control. More importantly, both HBx truncations could increase mSREBP-1 in the nuclear fractions (Fig. 7.2). As expected, wild-type HBx could up-regulate SREBP-1 and mSREBP-1 levels (Fig. 7.2). The nuclear fractions were prepared as described previously [137]. Antibodies for SREBP-1 (Santa Cruz Biotechnology), β-actin, and fibrillin (Sigma-Aldrich) were used in Western blot analysis. These results demonstrate that both HBx N-and C-terminal truncations can increase the amounts of SREBP-1 and mSREBP-1.

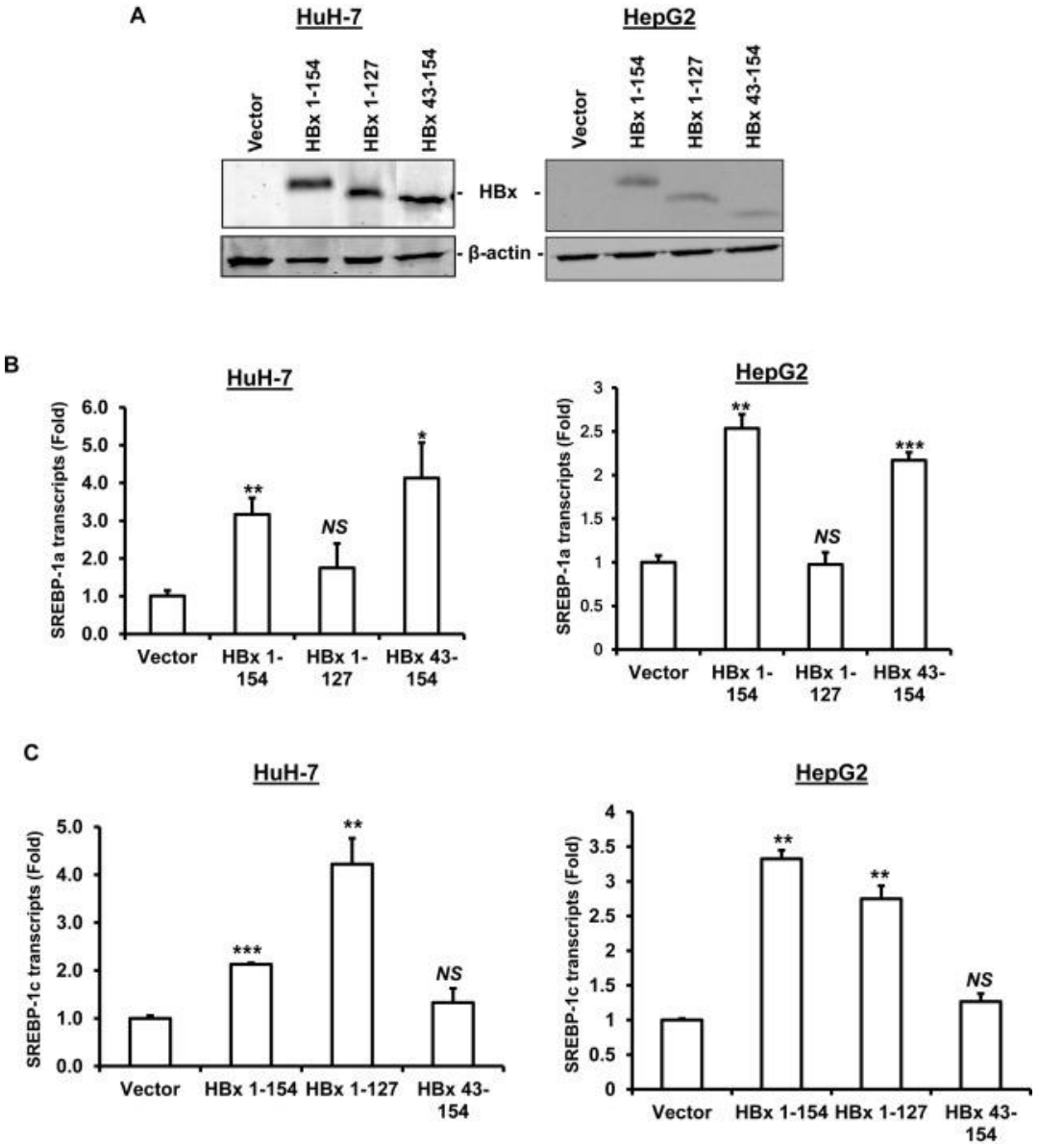

Fig. 7.1. Modulation of SREBP-1 transcripts by HBx truncations. Huh-7 and HepG2 cells were transfected with vector, plasmids expressing the full-length HBx 1-154, a C-terminally truncated (HBx 1-127) or a N-terminally truncated (HBx 43-154) mutants with an N-terminal Flag tag. (A). The expression of HBx proteins was analyzed by Western blotting using a Flag-specific antibody at 48 h after transfection. The levels of β-actin were also determined by a β-actin-specific antibody. This experiment was performed three times. (B and C). The transcript levels of SREBP-1a (B) or SREBP-1c (C) were determined by reverse transcription real-time PCR using gene-specific primers 16 h after transfection. The transcript level of β-glucuronidase was used for normalization. Statistical differences between samples were analyzed by the Student's *t* test and indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, or *NS* = not significant. These experiments were performed three times.

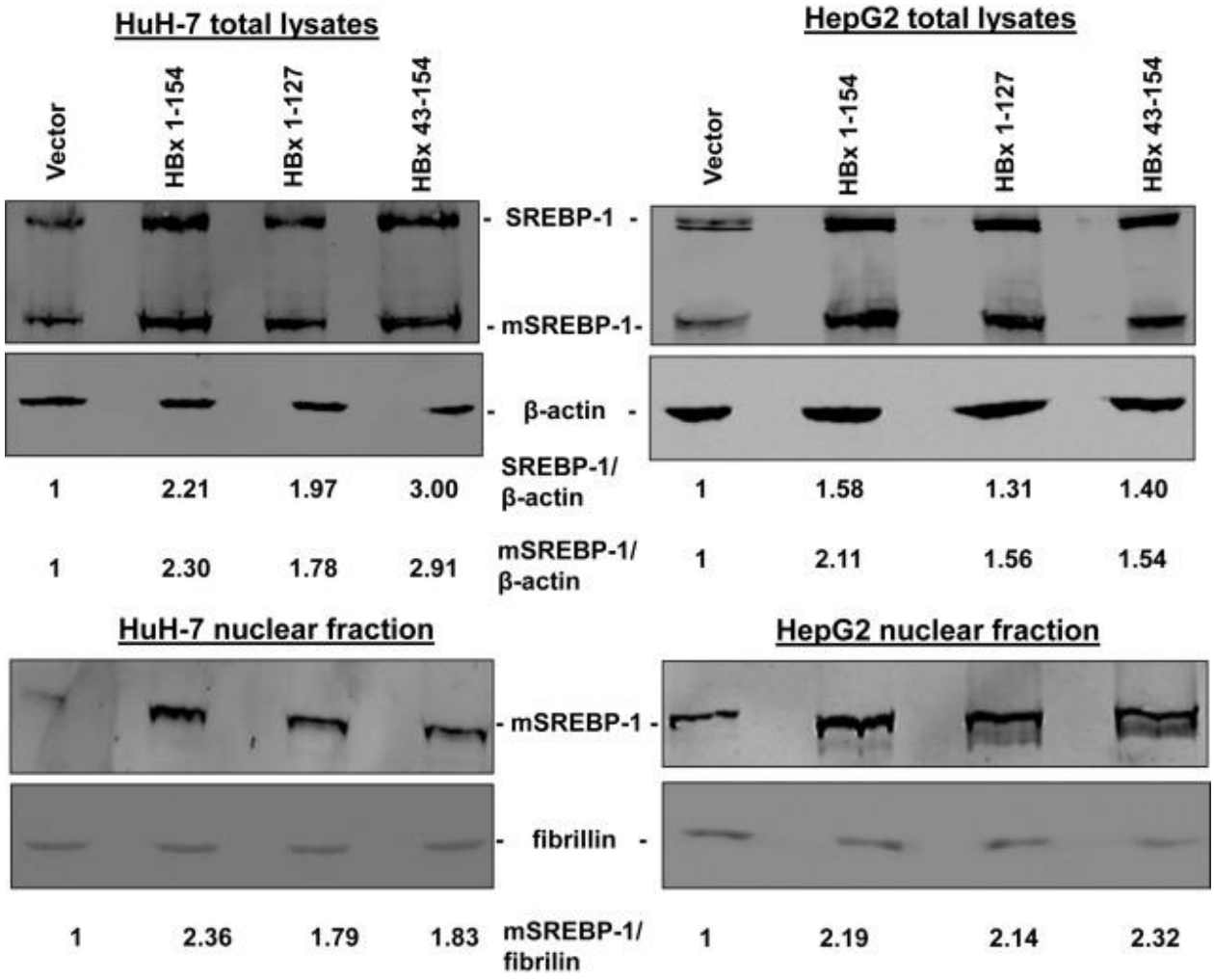

Fig. 7.2. Modulation of SREBP-1 at the protein level by HBx truncations. Huh-7 and HepG2 cells were transfected with vector, plasmids expressing the full-length HBx 1-154, or truncated HBx, HBx 1-127 and HBx 43-154. The protein levels of SREBP-1, mature SREBP-1 (mSREBP-1) in total lysates or in the nuclear fractions were analyzed by Western blotting using an SREBP-1-specific antibody at 48 h after transfection. The levels of β-actin or fibrillarin were also determined. Band intensities were analyzed by the Quantity One software (BioRad). The relative amounts of SREBP-1 or mSREBP-1 were calculated. This experiment was performed three times.

HBx activates HBV enhancers and thus increases HBV replication [11,12]. To examine how HBx truncations regulate HBV enhancers, we used HBV enhancer I/X promoter and HBV enhancer II/core promoter luciferase reporters and measured their activities after HBx expression in a luciferase reporter assay [137,237]. Luciferase levels were normalized to the protein concentration which was determined by the Bradford assay. The C-terminal truncation HBx 1-127 could up-regulate HBV enhancer I, but not HBV enhancer II (Fig. 7.3Aand B). In contrast, the N-terminal truncation HBx 43-154 could increase the activity of HBV enhancer II, but not HBV enhancer I (Fig. 7.3A and B). Wild-type HBx could up-regulate both enhancers as expected (Fig. 7.3A and B). These results demonstrate that HBx 1-127 and 43-154 have differential effects on the activities of HBV enhancers.

We previously showed that SREBP-1 is involved in HBV enhancer II activation by full-length HBx [137]. To determine the role of SREBP-1 in HBV enhancer activation by truncated HBx proteins, we knocked down SREBP-1 by a miRNA [137] and measured HBV enhancer activities after HBx expression. As shown in Fig. 7.3A and B, knocking down SREBP-1 negated the activation of both HBV enhancers by HBx 1-127 and HBx 43-154. These results demonstrate that SREBP-1 is involved in HBV enhancer activation by the truncated HBx proteins.

The activities of HBV enhancers are regulated by numerous transcription factors [225]. To gain insights on which transcription factors are involved in HBV enhancer activation by HBx 1-127 and 43-154, we generated two mutant HBV enhancer I constructs with the binding sequences of CREB (cAMP-response element-binding protein) and AP-1 mutated [238,239], as well as three HBV enhancer II mutants without the binding sequences for FXR (farnesoid X receptor) [202], C/EBP (CCAAT/enhancer-binding protein) [240], or Sp1 [241]. While HBx 1-154 and HBx 1-127 could still activate HBV enhancer I without the CREB binding sequence, albeit to a lower level than wild-type enhancer I, removal of the AP-1 site completely abolished HBV enhancer I activation by HBx proteins (Fig. 7.3C). These results suggest that the binding sequence for AP-1 is essential for HBV enhancer I activation by HBx 1-127.

Regarding HBV enhancer II, HBx and HBx 43-154 could increase the enhancer activity without the FXRE sequence (Fig. 7.3D), suggesting the nuclear receptor farnesoid X receptor was not essential. While removal of the C/EBP and the Sp1 binding sites reduced the activation of HBV enhancer II by full-length HBx, it completely negated HBV enhancer II up-regulation by

HBx 43-154 (Fig. 7.3D). These results suggest that C/EBP and Sp1 binding sequences are essential for HBV enhancer II up-regulation by HBx 43-154.

We next investigated the effects of HBx truncations on HBV transcription in HepG2 cells. We used plasmids containing a greater-than-unit-length HBV genomic DNA, wild-type (payw1.2) or a mutant without expressing HBx (payw1.2*7), with HBV endogenous regulatory elements [219]. Because we wanted to study the role of SREBP-1 in HBV transcription regulation by HBx, the effects of SREBP-1 knocking down and over-expression on HBV transcription were investigated. HBV transcription was determined by reverse transcription real-time PCR using HBV-specific primers HBV-FD (5' AGAAACAACACATAGCGCCTCAT 3') and HBV-rev (5' TGCCCCATGCTGTAGATCTTG 3') targeting the 3.5-kb pregenomic mRNA as previously described [196,237]. As shown in Fig. 7.4A and C, knocking down SREBP-1 by miRNA significantly reduced HBV transcript level in comparison to control miRNA, whereas expression of mSREBP-1a and mSREBP-1c significantly increased HBV mRNA levels. A Flag-tagged mSREBP-1a (aa. 1-517)-expressing plasmid was described previously [137] and a Flag-tagged mSREBP-1c (aa. 1-487) was amplified by PCR from the SREBP-1c cDNA (OriGene Technologies) and cloned into the pCMV2 Flag vector (Sigma-Aldrich). The expression of mSREBP-1a and mSREBP-1c proteins was confirmed in Western blotting using a Flag-specific antibody (Fig. 7.4E). These results suggest a role of both SREBP-1a and SREBP-1c in regulating HBV transcription.

To study the effects of HBx truncations on HBV transcription, we used a greater-than-unit-length HBV genomic DNA without expressing HBx (payw1.2*7) and determined HBV transcript levels after HBx expression. We found that both HBx 1-127 and HBx 43-154 could increase the HBV transcript level, which was abrogated after knocking down SREBP-1 (Fig. 7.4B). These results demonstrate that HBx truncations up-regulate HBV transcription in an SREBP-1-dependent manner.

To specifically investigate the roles of SREBP-1a and SREBP-1c in HBV transcription up-regulation by HBx truncations, we determined HBV mRNA levels after the expression of mSREBP-1a or mSREBP-1c. As shown in Fig. 7.4D, HBV mRNA levels were further increased by HBx in combination with either mSREBP-1a or mSREBP-1c expression, suggesting that the full-length HBx can up-regulate HBV transcription through both SREBP-1a and SREBP-1c. In contrast, the enhancement was only observed after mSREBP-1c expression for HBx 1-127 or

after mSREBP-1a expression for HBx 43-154 (Fig. 7.4B). These results demonstrate that both SREBP-1a and SREBP-1c are involved in HBV transcription up-regulation by the full-length HBx, but they have differential effects on HBV transcription up-regulation by the truncated HBx proteins.

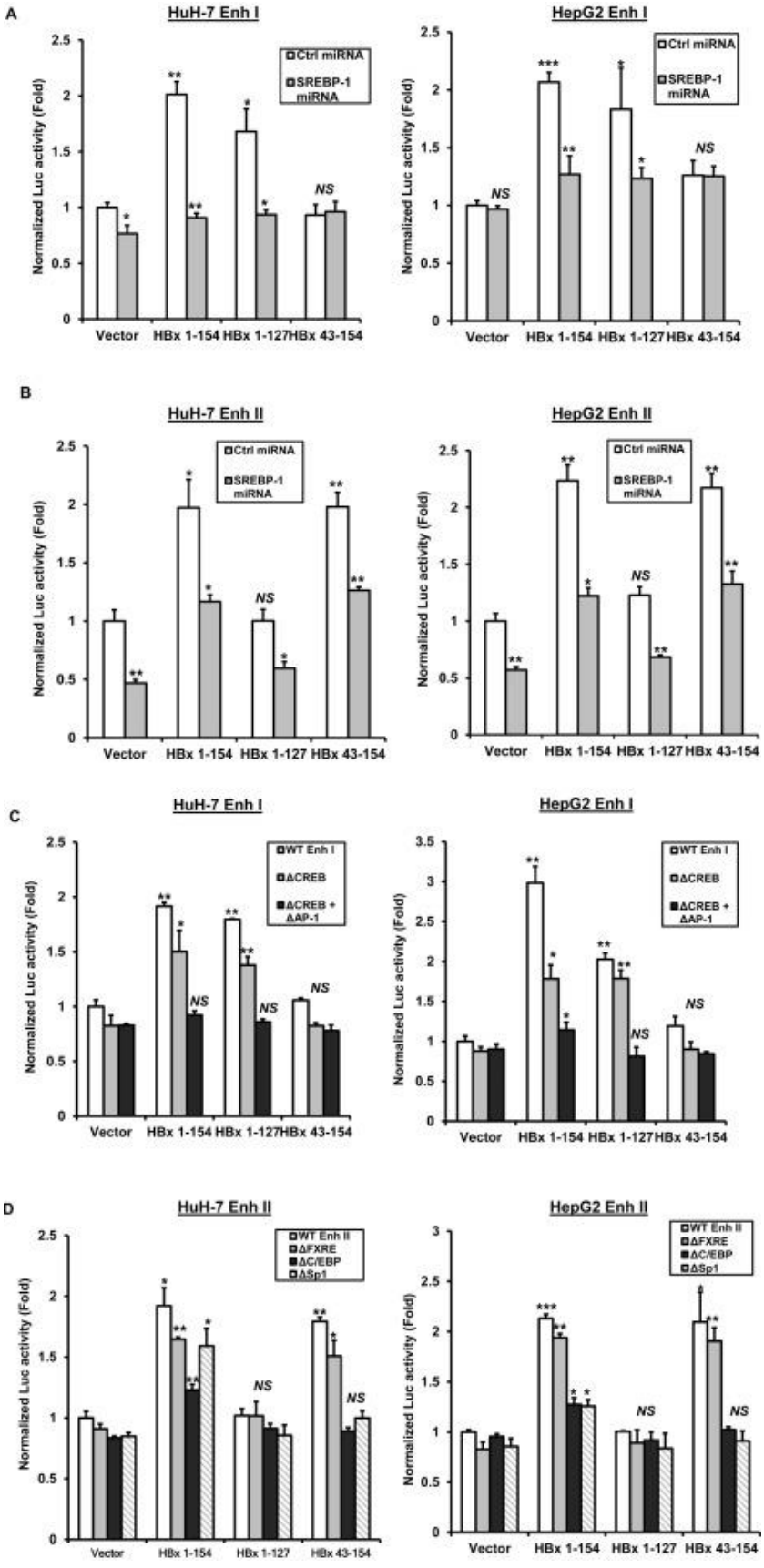
A
HuH-7 Enh I
HepG2 Enh I
Ctrl miRNA
SREBP-1 miRNA
Normalized Luc activity (Fold)
Vector
HBx 1-154
HBx 1-127
HBx 43-154
NS
B
HuH-7 Enh II
HepG2 Enh II
C
HuH-7 Enh I
HepG2 Enh I
WT Enh I
ΔCREB
ΔCREB + ΔAP-1
D
HuH-7 Enh II
HepG2 Enh II
WT Enh II
ΔFXRE
ΔC/EBP
ΔSp1

Fig. 7.3. Modulation of HBV enhancers by HBx truncations. Huh-7 and HepG2 cells were transfected with vector, plasmids expressing HBx 1-154, HBx 1-127, or HBx 43-154. (A and B). Cells were co-transfected with control or SREBP-1 miRNA, HBV enhancer I/X promoter (A) or HBV enhancer II/core promoter (B) luciferase reporters. (C and D).Cells were co-transfected with wild-type HBV enhancer I/X promoter or mutant promoters indicated (C) or wild-type HBV enhancer II/core promoter or mutant promoters indicated (D). At 48 h after transfection, luciferase activities were determined and normalized against the protein concentrations. Statistical differences between samples were analyzed by the Student's *t* test and indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, or *NS* = not significant. All experiments were performed three times.

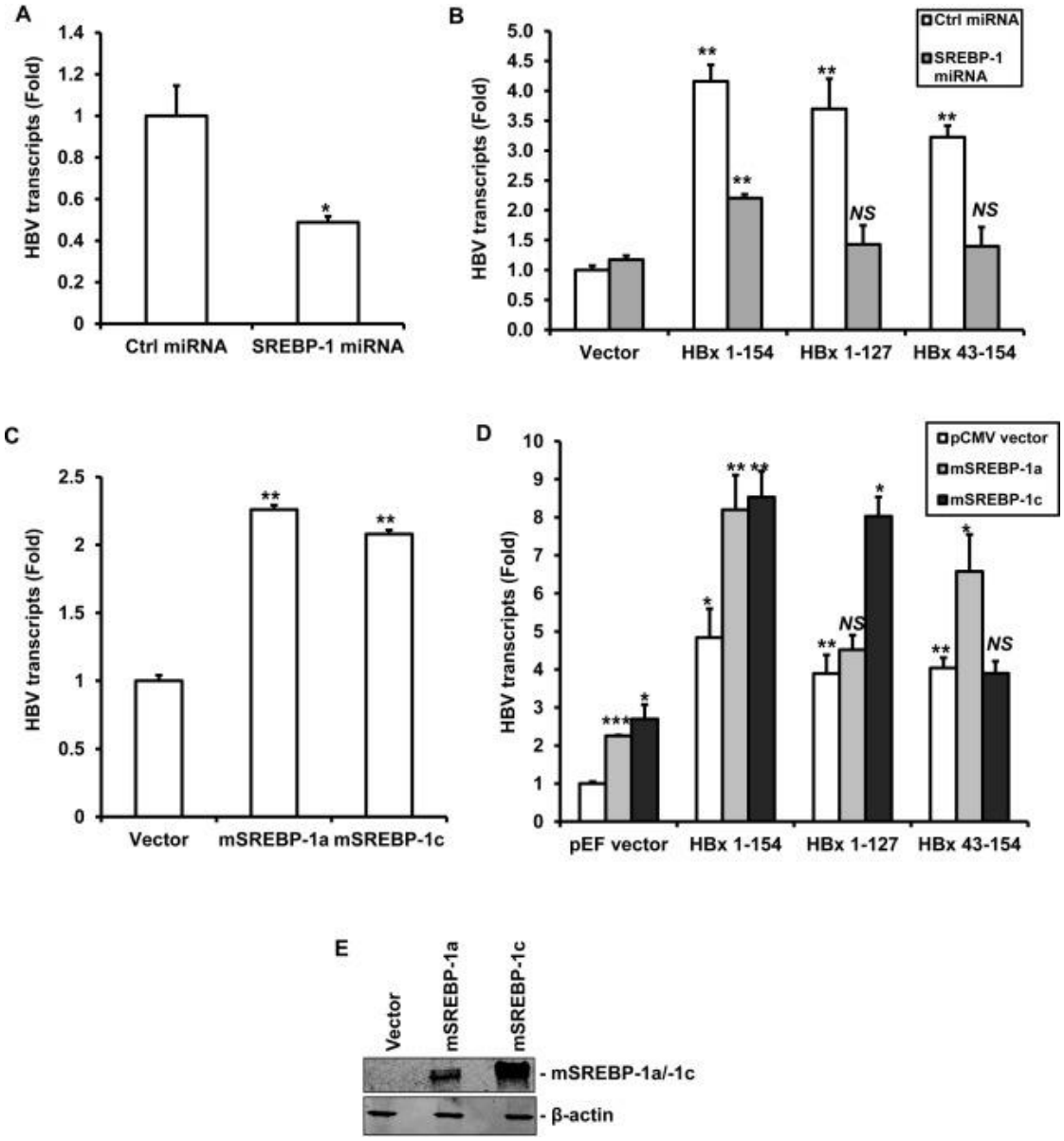

Fig. 7.4. Up-regulation of HBV transcription by HBx truncations dependent on SREBP-1. (A and C). HepG2 cells were co-transfected with an HBV genome plasmid pawy1.2, control or SREBP-1 miRNA (A), or vector, plasmids expressing mSREBP-1a or mSREBP-1c (C). (B and D). HepG2 cells were co-transfected with an HBV genome plasmid pawy1.2*7 (no HBx), vector or HBx-expressing plasmids, control or SREBP-1 miRNA (B), or vector, plasmids expressing mSREBP-1a or mSREBP-1c (D). At 48 h after transfection, HBV mRNA levels were determined by reverse transcription real-time PCR using HBV-specific primers. The transcript level of β-glucuronidase was used for normalization. Statistical differences between samples were analyzed by the Student's *t* test and indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, or *NS* = not significant. These experiments were performed three times. (E). Expression of mSREBP-1a and mSREBP-1c with a Flag-tag was analyzed by Western blotting with a Flag-specific antibody at 48 h after transfection. The levels of β-actin were also determined. This experiment was performed three times.

Finally, we studied the effects of HBx proteins on HBV replication in HepG2 cells. Capsid-associated HBV DNA was extracted and the HBV DNA copy number determined as per a published protocol [135]. As shown in Fig. 7.5A, expression of full-length HBx, HBx 1-127, and HBx 43-154 could significantly increase the HBV DNA levels. Knocking down SREBP-1 negated the up-regulation. These results suggest that HBx 1-127 and 43-154 retain the ability to up-regulate HBV replication, dependent on SREBP-1.

To further study the roles of two isoforms of SREBP-1 in HBV replication modulation by HBx, we once again expressed mSREBP-1a and mSREBP-1c. As shown in Fig. 7.5B, HBV DNA levels were further increased by full-length HBx in combination with either mSREBP-1a or mSREBP-1c expression, whereas this effect could only be observed for the combination of HBx 1-127 and mSREBP-1c or HBx 43-154 and mSREBP-1a. These results demonstrate that SREBP-1a and SREBP-1c are differentially involved in HBV replication up-regulation by the truncated HBx proteins.

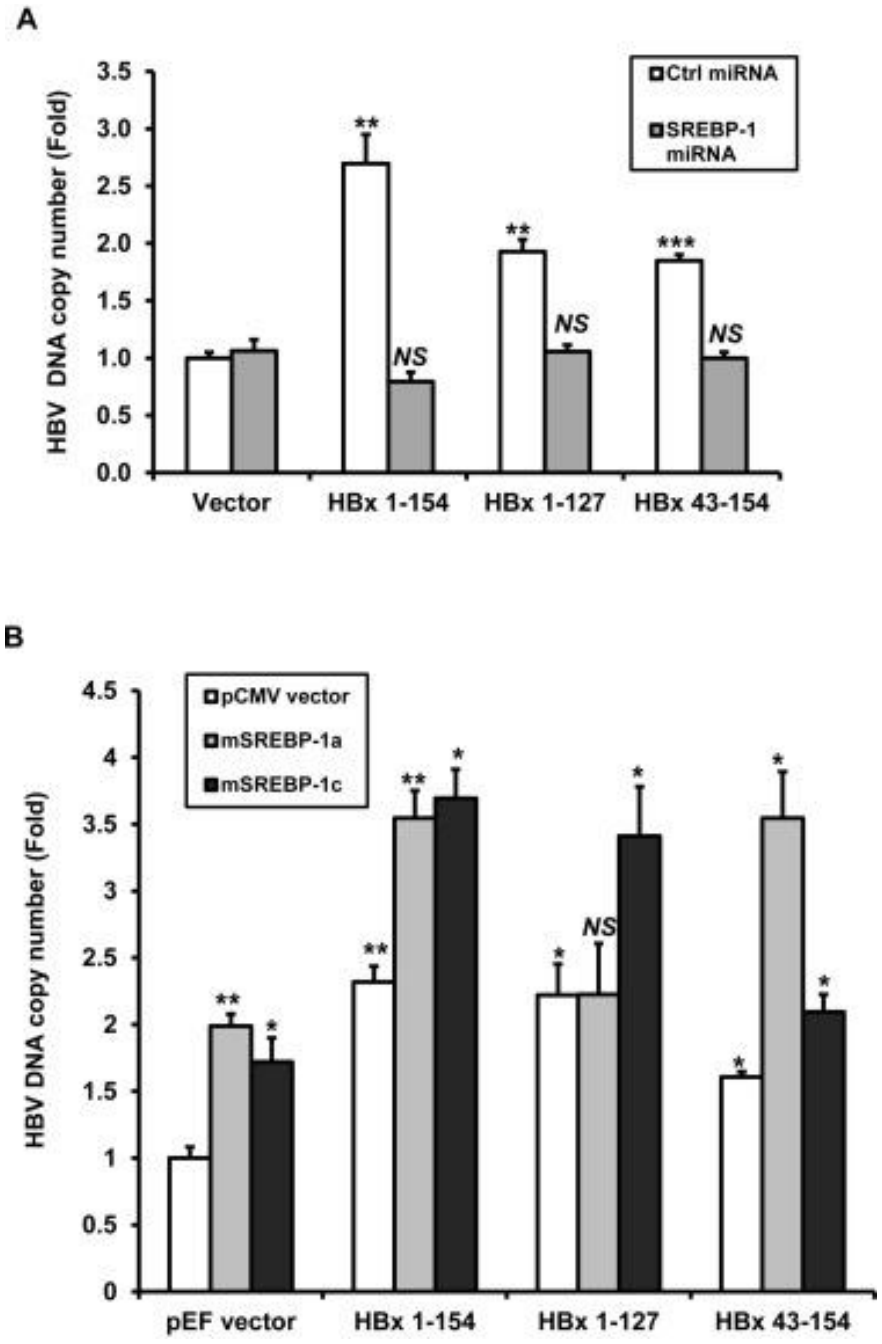

Fig. 7.5. Up-regulation of HBV replication by HBx truncations dependent on SREBP-1. HepG2 cells were co-transfected with an HBV genome plasmid pawy1.2*7 (no HBx), control or SREBP-1 miRNA (A), or vector, plasmids expressing mSREBP-1a ormSREBP-1c (B). At 48 h after transfection, capsid-associated HBV DNA was purified and its copy number determined by real-time PCR using HBV-specific primers. Statistical differences between samples were analyzed by the Student's *t* test and indicated as * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, or *NS* = not significant. These experiments were performed three times.

HBx plays an important role in the HBV life cycle and HBV pathogenesis [226]. Although different truncated forms of HBx have been consistently found in the tissues with chronic HBV infections [131,132,229,235,242,243], the pathobiological significance is poorly characterized. The worst outcome of chronic HBV infection is hepatocellular carcinoma and the abnormal lipogenesis mediated by SREBP-1 is believed to be a critical risk factor for carcinogenesis [18]. Therefore, understanding the modulation of SREBP-1 by HBx should have broad implications. It has been shown that HBx can up-regulate SREBP-1a and SREBP-1c transcription [110,137]. The results presented here further demonstrate that a C-terminally truncated HBx (HBx 1-127) can only up-regulate SREBP-1c transcription, while an N-terminally truncated HBx (HBx 43-154) can only up-regulate SREBP-1a transcription. Our previous work has identified that the transcriptional factors C/EBPα and E4BP4 are involved in SREBP-1a transcription modulation by HBx [137]. How HBx regulates SREBP-1c transcription has not been thoroughly studied. Because HBx can activate the activity of a nuclear receptor LXRα [110] and the SREBP-1c promoter contains the binding motif for LXRα [166], it is possible that HBx up-regulates SREBP-1c transcription through LXRα. Future studies should formally test this hypothesis and also investigate whether HBx truncation mutants retain the same mechanisms as the full-length HBx in modulating SREBP-1 transcription.

Activation of HBV enhancers is another important function of HBx. Our data showed that HBx 1-127 and HBx 43-154 can only respectively up-regulate either HBV enhancer I or enhancer II, dependent upon SREBP-1. We further showed that the binding sequences for AP-1, C/EBP, and Sp1 are involved in this process. In addition, we showed that HBx 1-127 and HBx 43-154 can up-regulate HBV transcription and replication. SREBP-1a can further enhance the activation by HBx 43-154 whereas SREBP-1c can do so for HBx 1-127.

Our study and those of others have suggested an important role of SREBP-1 in the functions of HBx and the HBV life cycle. The functions of SREBP-1 are exerted by the processed and mature form in the nucleus. The two isoforms, SREBP-1a and SREBP-1c, have common and unique functions through regulating shared or isoform-specific effector genes [68,244,245]. It will be interesting to determine the effector genes that are differentially modulated by the two HBx truncated mutants and understand their roles in HBV life cycle and pathogenesis.

In conclusion, we demonstrated that the N- and C-terminally truncated forms of HBx can differentially modulate SREBP-1a and SREBP-1c transcription, HBV enhancer I and HBV enhancer II activities. Furthermore, these two HBx mutants can activate HBV transcription and replication with SREBP-1a and SREBP-1c being differentially involved. Our research advances our understanding on the complex interactions between HBV and SREBP-1.

7.5 Acknowledgements

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8.0 THE ROLE OF PTEN IN HCV INFECTION

Data presented in Chapter 3 to 7 establishes that HBx enhances the activities of SREBP-1a and SREBP-1c which are involved in the up-regulation of HBV enhancers and thus contributes to HBV replication. In the following chapter, we will determine the mechanisms of how HCV infection regulates PTEN and how PTEN regulates HCV life cycle. Our lab has previously established that HCV core activates SREBP-1 and FASN through the PI3K/Akt pathway. PTEN is a suppressor of the PI3K/Akt pathway. Previous studies have been reported that HCV infection inhibits PTEN. Both HCV core and NS5A proteins can drive a feedback loop of NF-κB/PTEN/Akt which results in decreasing the PTEN protein level and contributes to HCC development. However, the mechanisms of how PTEN affects HCV infection needs to be further studied.

9.0 THE ROLE OF PTEN - HCV CORE INTERACTION IN HEPATITIS C VIRUS REPLICATION

Qi Wu[1], Paul Mellor[2], Yan Zhou[3], Deborah H. Anderson[2], and Qiang Liu[3,#]

[1]VIDO-InterVac and Department of Veterinary Microbiology, University of Saskatchewan,

[2]Cancer Research Group, University of Saskatchewan and Saskatchewan Cancer Agency,

[3]VIDO-InterVac, Department of Veterinary Microbiology and School of Public Health,

University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Running title: PTEN and core interaction inhibits HCV RNA replication

Keywords: Hepatitis C virus; core; PTEN; protein interaction; entry; replication; translation; secretion

Corresponding author.
VIDO-InterVac
University of Saskatchewan,
120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.
Fax: +1 306 966 7478.
E-mail address: qiang.liu@usask.ca. (Q. Liu).

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9.1 Authors' contribution

All of the experiments within this chapter were performed by Qi Wu, except Fig. 9.7E, which was performed by Paul Mellor. The manuscript was written by Qi Wu and edited by Qiang Liu and Deborah Anderson. The PTEN assay methods were written by Paul Mellor.

9.2 Abstract

Hepatitis C virus (HCV) infection leads to severe liver diseases including hepatocellular carcinoma (HCC). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor, is frequently mutated or deleted in HCC tumors. In this study, we determined the effects of PTEN on the HCV life cycle. We showed that PTEN inhibits HCV entry through its lipid phosphatase activity. PTEN has no effect on HCV RNA translation. PTEN decreases HCV replication and secretion and the protein phosphatase activity of PTEN is essential for this function. PTEN interacts with HCV core protein. HCV core aa. R50 in domain I and PTEN aa. 1-186 are essential for the interaction. PTEN no longer inhibits HCV replication in the absence of the interaction with the core protein. The interaction between PTEN and HCV core reduces PTEN levels which in turn regulates HCV replication. HCV core domain I protein increases the lipid phosphatase activity of PTEN in an *in vitro* assay, suggesting that HCV infection can also regulate PTEN. Taken together, our results demonstrated an important regulatory role of PTEN in the HCV life cycle.

9.3 Introduction

More than 185 million people are estimated to be infected by hepatitis C virus (HCV) worldwide [246]. HCV infection leads to severe liver diseases such as steatosis, cirrhosis and hepatocellular carcinoma (HCC). HCV is an enveloped positive-sense single-stranded RNA virus in the *Flaviviridae* family [247]. HCV has a genome of about 9,600 nucleotides, consisting of two untranslated regions (UTRs) at both ends of the genome and a large open reading frame. HCV life cycle begins with entry into the target cells through numerous cellular receptors. Then

the HCV genomic RNA is translated into a polyprotein through an internal ribosomal entry site (IRES) within the 5'-UTR [248]. The polyprotein is processed by cellular and viral proteases to generate structural and non-structural proteins. The endoplasmic reticulum membrane is modified by viral and cellular factors to generate a membranous web that is the major site of viral RNA replication. The newly synthesized RNAs are packaged into nucleocapsids. Viral particle maturation and secretion are achieved after envelope acquisition. HCV core is a structural protein that forms the viral nucleocapsid. In addition, HCV core has regulatory functions in cell signaling and lipid metabolism [249]. It has been reported that HCV core is associated with the development of HCC in a transgenic mouse model [143].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase which contains both lipid and protein phosphatase activities. PTEN contains a short N-terminal PtdIns(4,5)P_2-binding domain (PBD) (aa. 1-6), a phosphatase domain (aa. 7-185), a C2 domain (aa. 186-351), and a C-terminal tail containing PEST (Pro, Glu, Ser, Thr) sequences (aa. 352-401) and a postsynaptic-density protein of 95 kDa, discs large, PDZ domain-interaction motif (aa. 401-403) [45,250]. PTEN acts as a tumor suppressor by down-regulating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, one of the most critical cancer-promoting pathways [45]. PTEN is frequently mutated or deleted in tumors including HCC [45,49]. Two naturally occurring mutations in the phosphatase domain disrupt the phosphatase activities of PTEN: C124S mutation abrogates both lipid and protein phosphatase activities; and G129E mutation abrogates lipid phosphatase activity only. The Y138L mutation, identified in 2010, abrogates protein phosphatase activity only [50]. Of note, the human hepatoma cell line Huh-7 that is widely used in HCV research contains a functional PTEN [251,252].

It has been reported that HCV activates the PI3K/Akt pathway with the involvement of core, NS4B, and NS5A proteins [161,164,253,254]. Consequently, the PI3K/Akt pathway enhances HCV entry, translation, and replication [255-258]. However, the possible influence of PTEN on the HCV life cycle has not been well characterized. A very early study showed that HCV infection increases PTEN phosphorylation with no effect on the total PTEN level [160]. In contrast, the Kumar group showed that HCV infection is associated with less nuclear PTEN which favors HCV replication [158]. Additional studies demonstrated that HCV core and NS5A proteins can down-regulate PTEN protein levels [147,149,156,259]. As for the effects of PTEN on HCV, PTEN has been shown to down-regulate HCV secretion with no effect on HCV RNA

replication [259]. Together, the exact role of PTEN in HCV life cycle is not clear and requires further investigation.

In this study, we showed that PTEN inhibits HCV entry, replication and secretion, but has no effect on HCV RNA translation. HCV core protein interacts with PTEN through domain I. The interaction between HCV core and PTEN decreases the level of PTEN and likely contributes to HCV replication inhibition by PTEN. The presence of HCV core domain I protein enhances the lipid phosphatase activity of PTEN, suggesting mutual regulatory processes. These results demonstrated a broad regulatory role of PTEN in the HCV life cycle.

9.4 Materials and Methods

9.4.1 Plasmid constructs and *in vitro* transcription

Plasmids for generating lentiviral particles, pMD2.G, psPAX2, pCMV-HCV-2a J6 core-E1-E2, and pTRIP-CMV-Luc-puro, were described previously [164,221]. The pGIPZ lentiviral plasmid expressing PTEN-specific shRNA with the target sequence 5' GAGACAGACTGATGTGTAT 3' located in the 3'-UTR of PTEN mRNA and non-silencing control shRNA were purchased from Open Biosystems. Inducible PTEN shRNA was constructed by transferring the shRNA sequence into a Tet-On pTRIPZ lentiviral vector (Open Biosystems). Plasmid expressing PTEN with an N-terminal Flag-tag, pCMV5 Flag-Human PTEN, was received from Dr. Jack Dixon [260] and used to generate plasmids expressing truncations and point mutations of PTEN: aa. 1-185, aa. 186-403, C124S, G129E, Y138L [259]. We also constructed PTEN-expressing plasmids with a 3xFlag-tag or GST fusion in the pEF-cyto-myc vector for use in some experiments. To allow the expression of the PTEN protein from the wild-type and shRNA-sensitive transcript, the 3'-UTR sequence of the PTEN mRNA, 1000 bps in length, was amplified by reverse transcription - PCR using RNA extracted from Huh-7 cells and cloned 3' to the coding sequence of PTEN, generating plasmid PTEN-3'-UTR WT. The target sequence of the shRNA was then removed from the 3'-UTR by site-directed mutagenesis, resulting in a shRNA-resistant PTEN-expressing plasmid PTEN-3'-UTR Mut. The wild-type and mutant 3'-UTR sequences were also cloned into the RNA interference vector psiCHECK-2, a gift

from Dr. Robert Blelloch (Addgene plasmid # 31882) [261]. Plasmids expressing GST-PTEN fusion proteins in the pGEX-6P-1 vector were described previously [262]. Plasmid pFLneo-J6/JFH-1 (p7-rLuc-2A) containing the full-length HCV-2a J6/JFH-1 genomic sequence with an internal renilla luciferase reporter gene and plasmid pFLneo-J6/JFH-1 (p7-rLuc-2A) GNN, the replication-deficient version, were obtained from Dr. Charles Rice [263]. Plasmid HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) was constructed by insertion of a Flag-tag between the amino acids S2 and T3 of the core protein as previously described [264]. Plasmid HCV-2a J6 core-R50A Flag/JFH-1 (p7-rLuc-2A) contains the R50A mutation [264]. A subgenomic replicon plasmid pSGR-rLuc-Neo-HCV-2a JFH-1 NS3-5B with rLuc reporter was constructed as previously described [265]. A monocistronic HCV RNA translation reporter pT7 HCV-2a JFH-1 5'-UTR-core^{aa1-16}-Luc2-NS5B^{5}-3'-UTR was constructed as described [51,257,266] using the JFH-1 sequence from the plasmid pHCV-2a JFH-1_pUC provided by Dr. Takaji Wakita [267]. To generate a plasmid expressing HCV core protein, the core coding sequence was amplified by PCR using the plasmid pFLneo-J6/JFH-1 (p7-rLuc-2A) as template and cloned into the pEF vector. Plasmids expressing the individual domains (as EGFP fusions), truncations, or point mutations of HCV core were constructed. To allow protein expression in *E. coli*, the coding sequences for HCV core, full-length or domain I with or without R50A mutation, were cloned into the pT7 His_6-SUMO vector (Lucigen). BFP (blue fluorescent protein)-expressing plasmid in the pT7 His_6-SUMO vector was described previously [268]. All plasmids were constructed using standard methods and confirmed by DNA sequencing. HCV genomic RNAs and translation reporter RNAs were generated by *in vitro* transcription using the MEGAscript T7 *In Vitro* Transcription reagents (Ambion).

9.4.2 Cell culture, transfection, and generation of stable cell lines

Huh-7 cells, Huh-7.5 cells and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Cells were transfected with plasmid DNA or HCV RNA using the calcium phosphate precipitation method [163] or the DMRIE-C reagent (Thermo Fisher Scientific), respectively. For establishing HCV replicating cells, Huh-7 cells were transfected with HCV RNA and selected by G418 (Enzo Life Sciences) [164].

9.4.3 Luciferase and MTT assays

For luciferase assay, cells were lysed in Passive Lysis Buffer (Promega) and the firefly or renilla luciferase activities were measured by Luciferase Assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Firefly or renilla luciferase levels were normalized to the protein concentrations determined by the Bradford assay (Bio-Rad Laboratories). Cell viability MTT assay was performed as previously described [266]. These assays were performed at least three times and the results analyzed for statistical differences by the Student's *t* test. A p value of ≤ 0.05 was considered statistically significant.

9.4.4 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed as previously described [137]. RNA was isolated from cells with TriZol (Thermo Fisher Scientific) followed by DNase I (Thermo Fisher Scientific) digestion. Reverse transcription was carried out by Superscript II (Thermo Fisher Scientific) and random priming. HCV RNA levels were determined by real-time RT-PCR using HCV-specific primers HCV-FD (5' AGAGCCATAGTGGTCTGCGGAAC 3') and HCV-rev (5' CCTTTCGCAACCCAACGCTACTC 3') [269]. The transcript levels of GUSB, a house keeping gene, were used for normalization [137]. Each sample was quantified in triplicates. Relative changes in RNA levels were analyzed by the $2^{-\Delta\Delta ct}$ method using the iQ5 program (Bio-Rad Laboratories).

9.4.5 HCV entry, replication, translation and secretion assays

HCV entry was determined using HCV lentiviral pseudoparticles carrying the luciferase reporter gene (HCVpp-Luc) as previously described [221]. The replication of HCV-2a J6/JFH-1 (p7-rLuc-2A) RNA in Huh-7 cells harboring HCV replicating RNA or after infection with the HCV-2a J6/JFH-1 (p7-rLuc-2A) virus was analyzed by determining the renilla luciferase activity by luciferase assay or HCV RNA levels by real-time PCR. HCV-2a J6/JFH-1 (p7-rLuc-2A) virus was collected from the supernatant of Huh-7 cells harboring HCV-2a J6/JFH-1 genomic RNA

and titrated by focus forming assay as described [263]. HCV RNA translation assay was performed as previously described [258]. To determine the effects of PTEN on HCV secretion, Huh-7 cells with HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were transfected with plasmids expressing PTEN, wild-type or phosphatase mutants. At 48 hours after transfection, intracellular HCV RNA levels were determined by luciferase assay and recorded as "donor levels". At the same time, virus-containing supernatants were collected and used for infecting native Huh-7.5 cells. At 72 hours after infection, luciferase assay was preformed to determine the levels of HCV RNA and normalized against the corresponding "donor" levels.

9.4.6 GST pull-down, co-immunoprecipitation (co-IP), and Western blotting

GST pull-down assay was performed using cell lysates in radioimmunoprecipitation assay (RIPA) buffer and Glutathione Sepharose 4B (GE Healthcare) according to a standard protocol [262]. For Flag co-IP experiments, cells were harvested by RIPA buffer and incubated with an anti-Flag (Sigma-Aldrich) antibody at 4 ℃ overnight and then incubated with Protein G Sepharose (GE Healthcare) at 4 ℃ for 4 hours. The mixtures were centrifuged at 10,000 rpm for 10 minutes and the supernatants removed. The pellets were resuspended in a lysis buffer containing SDS and boiled for 10 minutes to elute the proteins. For Western blotting, proteins were subjected to SDS-PAGE and then blotted onto nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBS and incubated with a primary antibody overnight at 4 ℃. Membranes were washed and incubated with a secondary antibody for 1 hour at room temperature. After a wash with PBST (PBS+0.1% Tween 20), membranes were scanned using Li-Cor Odyssey scanner (ODY-CLx) and band intensities were determined by the Quantity One software (Bio-Rad Laboratories). The antibodies used were: HCV core (Anogen), GST (Cell Signaling Technology, CST), PTEN (CST), β-actin (CST), Flag (Sigma-Aldrich), His_6-tag (Roche), and secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

9.4.7 Purification of recombinant proteins

Expression of recombinant proteins in *E. coli* was induced by IPTG (Thermo Fisher Scientific). GST-PTEN fusion proteins were purified by Glutathione Sepharose 4B (GE Healthcare). For use in PTEN phosphatase assay, the PTEN protein was cleaved from GST using the PreScission protease (GE Healthcare) as previously described [270]. His_6-tagged HCV core and PTEN proteins were purified by Ni-NTA agarose (Qiagen).

9.4.8 PTEN lipid phosphatase activity assay

The lipid phosphatase activity of PTEN in the presence of HCV core domain I protein was measured using the PTEN Activity ELISA kit (Echelon Biosciences) according to manufacturer's instructions. Briefly, 20 nM of PTEN was incubated with 0 - 2.852 μM of HCV core domain I proteins, wild-type or R50A mutant, at 37 °C for 5 minutes prior to the addition of 16 μM of the PtdIns(3,4,5)P_3 substrate at 37°C for 1 hour. Activity was detected using a SpectraMax M5 spectrophotometer (Molecular Devices) at 450 nm and presented as the percentage increases in the presence of HCV core domain I proteins compared to PTEN alone.

9.5 Results

9.5.1 PTEN negatively regulates HCV RNA levels after HCV infection

Although the mutual interactions between the PI3K/Akt pathway and HCV are well documented, the effect of PTEN, a negative regulator of the PI3K/Akt pathway, on HCV is less clear [161,164,253-258]. We therefore started off by determining the effect of PTEN on HCV RNA levels after HCV infection. As shown in Fig. 9.1A, overexpression of full-length PTEN, or the aa. 1-185 fragment, but not aa. 186-403, significantly reduced HCV RNA levels. Because the aa. 1-185 fragment contains the phosphatase domain of PTEN, these data indicate that the phosphatase activities are required for inhibiting HCV RNA accumulation after infection. To test this idea, we used three phosphatase-deficient mutants. The lipid phosphatase deficient G129E

mutant behaved the same as wild-type, whereas the protein phosphatase deficient Y138L and the lipid and protein phosphatase deficient C124S mutants had no effect (Fig. 9.1A). The expression of wild-type or mutant PTEN proteins, demonstrated by Western blotting (Fig. 9.1B), had no effects on cell viability as measured by MTT assay (Fig. 9.1C).

To confirm the inhibitory effects of PTEN on HCV, we wanted to determine whether decreasing PTEN enhances HCV RNA levels. We used an inducible shRNA targeting the 3'-UTR of PTEN to achieve PTEN knockdown. We first determined the efficiency and specificity of the PTEN shRNA using a bicistronic RNA interference reporter system. As shown in Fig. 9.1F, PTEN shRNA significantly decreased the luciferase activity linked to wild-type PTEN-3'-UTR in comparison to non-silencing control shRNA, indicating an effective knockdown. In contrast, PTEN shRNA did not affect the luciferase activity linked to a mutant PTEN-3'-UTR without the target sequence of the shRNA. These data indicate that the shRNA can effectively and specifically reduce PTEN levels. We then generated Huh-7.5 cells that express the PTEN shRNA or non-silencing control shRNA upon induction with Doxycycline.

Expression of PTEN shRNA resulted in significantly higher HCV RNA level after HCV infection than control shRNA (Fig. 9.1D, compare Vector transfection in Ctrl and PTEN shRNA cells). To demonstrate whether the altered HCV RNA level detected was specifically due to changes in PTEN levels, the inducible knockdown cell lines were transfected with increasing amounts of plasmids expressing PTEN-3'-UTR WT, or shRNA resistant PTEN-3'-UTR Mut. As shown in Fig. 9.1D, increasing the levels of PTEN protein after PTEN-3'-UTR WT or PTEN-3'-UTR Mut transfection in Ctrl shRNA cells was associated with decreasing HCV RNA levels. In contrast, increasing the levels of PTEN protein after PTEN-3'-UTR WT transfection in PTEN shRNA cells had no effect, whereas transfection with PTEN-3'-UTR Mut in PTEN shRNA cells decreased HCV RNA levels in a dose-dependent manner (Fig. 9.1D). The overall PTEN levels in these cells were determined by Western blotting using a PTEN-specific antibody (Fig. 9.1D). Knocking down PTEN had no effects on cell viability (Fig. 9.1E). Taken together, these data indicate that PTEN decreases HCV RNA levels after HCV infection dose-dependently.

Because the HCV RNA levels measured may represent the combined effects of PTEN on HCV entry, replication, and translation, it is therefore necessary to study the effect of PTEN on each of these steps in HCV life cycle.

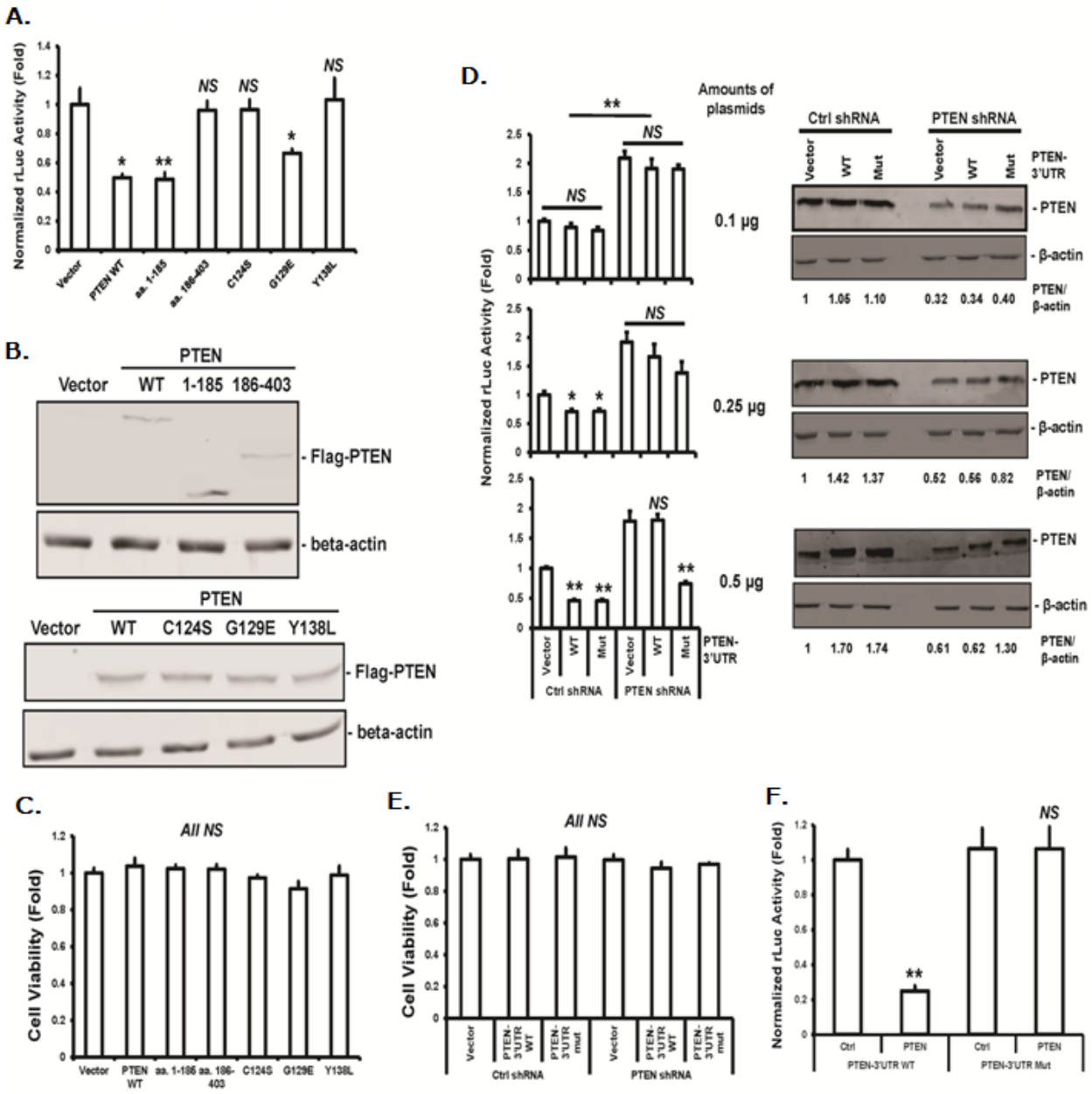
A.
Normalized rLuc Activity (Fold)
NS
Vector
PTEN WT
aa. 1-185
aa. 186-403
C124S
G129E
Y138L
B.
PTEN
Vector
WT
1-185
186-403
- Flag-PTEN
- beta-actin
PTEN
Vector
WT
C124S
G129E
Y138L
- Flag-PTEN
- beta-actin
C.
All NS
Cell Viability (Fold)
Vector
PTEN WT
aa. 1-185
aa. 186-403
C124S
G129E
Y138L
D.
Amounts of plasmids
0.1 μg
0.25 μg
0.5 μg
Normalized rLuc Activity (Fold)
Ctrl shRNA
PTEN shRNA
PTEN-3'UTR
Vector
WT
Mut
- PTEN
- β-actin
1 1.05 1.10 0.32 0.34 0.40
1 1.42 1.37 0.52 0.56 0.82
1 1.70 1.74 0.61 0.62 1.30
PTEN/β-actin
E.
All NS
Cell Viability (Fold)
Vector
PTEN-3'UTR WT
PTEN-3'UTR mut
Ctrl shRNA
PTEN shRNA
F.
Normalized rLuc Activity (Fold)
NS
Ctrl
PTEN
PTEN-3'UTR WT
PTEN-3'UTR Mut

Fig. 9.1. PTEN protein phosphatase activity is required for decreasing HCV RNA level after HCV infection. (A) Huh-7.5 cells were transfected with plasmids expressing wild-type Flag-PTEN (PTEN WT), truncated mutants, phosphatase deficient mutants, or empty vector. Forty eight hours after transfection, cells were infected with HCV-2a J6/JFH-1 (p7-rLuc-2A) virus at a multiplicity of infection (MOI) of 1. Seventy two hours post-infection (hpi), luciferase assay was performed using the cell lysates. This experiment was performed in triplicate. (B). The protein levels of PTEN proteins and β-actin after transfection were determined by Western blotting using anti-Flag and anti-β-actin antibodies, respectively. (C). Cell viability was determined by MTT assay 72 hpi. This experiment was performed in triplicate. (D). Huh-7.5 cells expressing an inducible PTEN shRNA or non-silencing control shRNA were treated with 1 μg/mL of Doxycycline for 48 hours. Cells were then infected with HCV-2a J6/JFH-1 (p7-rLuc-2A) virus at an MOI of 1. At 24 hpi, cells were transfected with increasing amounts of plasmids expressing Flag-tagged PTEN with wild-type or mutant 3'-UTR, or empty vector. Luciferase assay was performed 48 hours after transfection (left panel). In the right panel, the protein levels of PTEN and β-actin at 36 hours after transfection were determined by Western blotting using anti-PTEN and anti-β-actin antibodies, respectively. Relative PTEN band intensities against β-actin were given underneath each sample. This experiment was performed in duplicate. (E). Cell viability was determined by MTT assay in parallel with luciferase assay. This experiment was performed in duplicate. (F). Huh-7 cells were co-transfected with plasmids expressing non-silencing control shRNA or shRNA targeting the 3'-UTR of PTEN, together with bicistronic RNA interference reporter plasmids with (PTEN-3'-UTR wt) or without (PTEN-3'-UTR mut) the shRNA target sequence. Dual luciferase assay was performed 48 hours after transfection. The renilla luciferase (rLuc) activity representing the knockdown efficiency was normalized against firefly luciferase activity driven by a constitutive promoter on the reporter plasmid. Luciferase activities were expressed as fold changes relative to vector control. The statistical differences between samples were demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, or *NS* for not significant. This experiment was performed in triplicate.

9.5.2 PTEN lipid phosphatase activity inhibits HCV entry

HCV entry is the first step in the HCV life cycle. To study the effect of PTEN on HCV entry, we used the HCVpp approach. As shown in Fig. 9.2A, overexpression of full-length PTEN (PTEN WT) and the aa. 1-185 fragment significantly inhibited HCVpp entry, whereas aa. 186-403 had no effect, suggesting a role of the phosphatase activities in this process. To test this, we once again used three phosphatase-deficient mutants. The protein phosphatase deficient Y138L mutant inhibited HCVpp entry as effectively as the wild-type PTEN, whereas the lipid phosphatase deficient G129E and the lipid and protein phosphatase deficient C124S mutants had no effect on HCV entry (Fig. 9.2A). These data collectively indicate that PTEN lipid phosphatase but not protein phosphatase activity is required for the inhibition of HCV entry. The expression of wild-type or mutant PTEN proteins was demonstrated by Western blotting (Fig. 9.2B).

To further determine the effect of PTEN on HCV entry, we measured HCV entry after knocking down PTEN levels. We also determined whether the observed effects were indeed due to PTEN, and not an off-target effect of the shRNA used. To this end, inducible PTEN knockdown and non-silencing control Huh-7.5 cells were transfected with vector, or plasmids expressing PTEN cDNA linked to wild-type 3'-UTR or mutant 3'-UTR without the shRNA target sequence. After induction with Doxycycline, cells were infected with HCVpp-Luc. As shown in Fig. 9.2C, knocking down endogenous PTEN by shRNA significantly enhanced HCV entry (compare HCV entry after Vector transfection in Ctrl and PTEN shRNA cells). Overexpression of wild-type PTEN in Ctrl shRNA cells significantly decreased HCV entry, but exhibited no inhibitory effect in PTEN shRNA cells. More importantly, expression of PTEN which could not be knocked down by shRNA significantly inhibited HCV entry in PTEN shRNA-expressing cells. The overall PTEN levels in these cells were determined by Western blotting using a PTEN-specific antibody (Fig. 9.2D). Taken together, these data indicate an inverse relationship between PTEN levels and HCV entry.

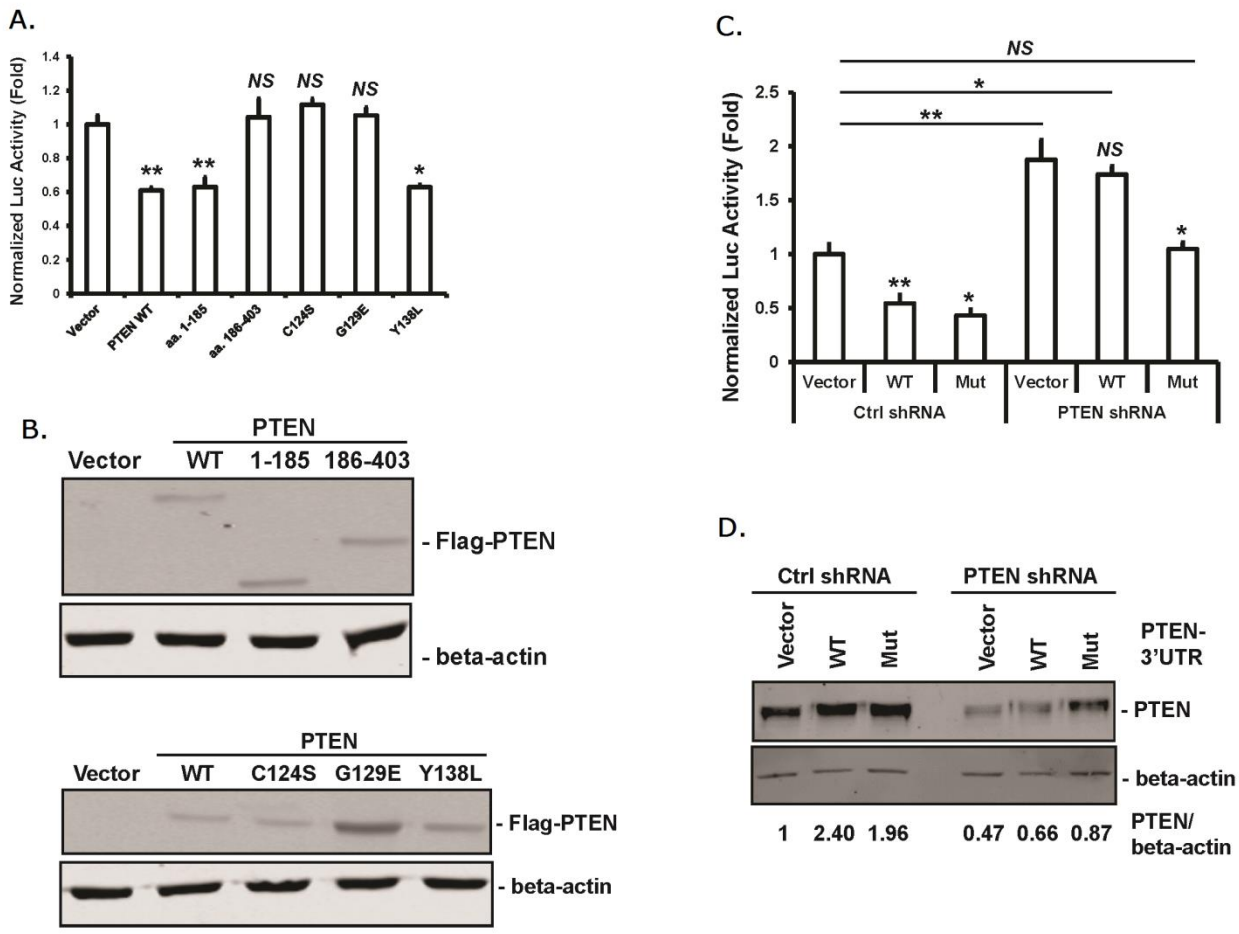

Fig. 9.2. PTEN inhibits HCV entry through its lipid phosphatase activity. (A). Huh-7.5 cells were transfected with plasmids expressing wild-type Flag-PTEN (PTEN WT), truncated mutants, phosphatase deficient mutants, or empty vector. Forty eight hours after transfection, cells were infected using HCV-2a J6 pseudoparticles with a firefly luciferase reporter (HCVpp-Luc). Luciferase activity was measured 24 hours after infection and normalized to total protein amount. Luciferase activities were expressed as fold changes relative to vector control. Statistical differences between samples were demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, or *NS* for not significant. This experiment was performed in triplicate. (B). The protein levels of Flag-tagged PTEN after transfection were determined by Western blotting using an anti-Flag antibody. The β-actin levels were determined by blotting cell lysates with a β-actin antibody as loading controls. (C and D). Huh-7.5 cells expressing inducible PTEN shRNA or non-silencing control shRNA were transfected with plasmids expressing Flag-tagged PTEN with wild-type or mutant 3'-UTR, or empty vector. Cells were treated with 1 μg/mL of Doxycycline for 48 hours. Cells were then infected with HCVpp-Luc and luciferase activity was determined 24 hours after infection (C). This experiment was performed in triplicate. In (D), the protein levels of PTEN and β-actin in Huh-7.5 cells were determined by Western blotting using anti-PTEN and anti-β-actin antibodies, respectively. Relative PTEN band intensities against β-actin were given underneath each sample.

9.5.3 PTEN protein phosphatase activity is required for the inhibition of HCV replication

To study whether PTEN regulates HCV replication, we determined the level of HCV replication in HCV genomic replicon cells upon ectopic expression of PTEN and its mutants by transfection. As shown in Fig. 9.3A, overexpression of full-length PTEN, or the aa. 1-185 fragment, but not aa. 186-403, significantly inhibited HCV replication. These data indicate that the phosphatase domain of PTEN is required for inhibiting HCV replication. We further observed that PTEN with G129E mutation, but not C124S or Y138L mutations, inhibited HCV replication (Fig. 9.3A). The expression of wild-type or mutant PTEN proteins was demonstrated by Western blotting (Fig. 9.3B). These data suggest that the protein phosphatase activity of PTEN is essential for the inhibition of the replication of HCV genomic RNA. To study the roles of viral structural and non-structural proteins in HCV replication inhibition by PTEN, we determined the effects of PTEN on the replication of an HCV subgenomic replicon. Interestingly, we found that PTEN did not inhibit HCV replication in the absence of HCV structural proteins (Fig. 9.3C). These data collectively indicate that PTEN inhibits HCV replication with the possible involvement of viral structural proteins.

9.5.4 PTEN does not affect HCV RNA translation

To examine whether PTEN affects HCV RNA translation, we used a well-established system that we reported previously [258,266]. Huh-7 cells were co-transfected with a monocistronic HCV translation rLuc reporter RNA (Fig. 9.4A) or HCV genomic RNA that is replication-deficient (Fig. 9.4C), together with plasmid vector or PTEN-expressing plasmid. No significant changes in RNA translation were observed in both cases, suggesting that PTEN has no effect on HCV RNA translation. To confirm these results, the monocistronic HCV translation rLuc reporter (Fig. 9.4B) or genomic RNAs (Fig. 9.4D) were transfected into the inducible PTEN knockdown cells or non-silencing control cells. Knocking down PTEN did not affect HCV RNA translation. These data indicate that PTEN has a little effect on HCV RNA translation.

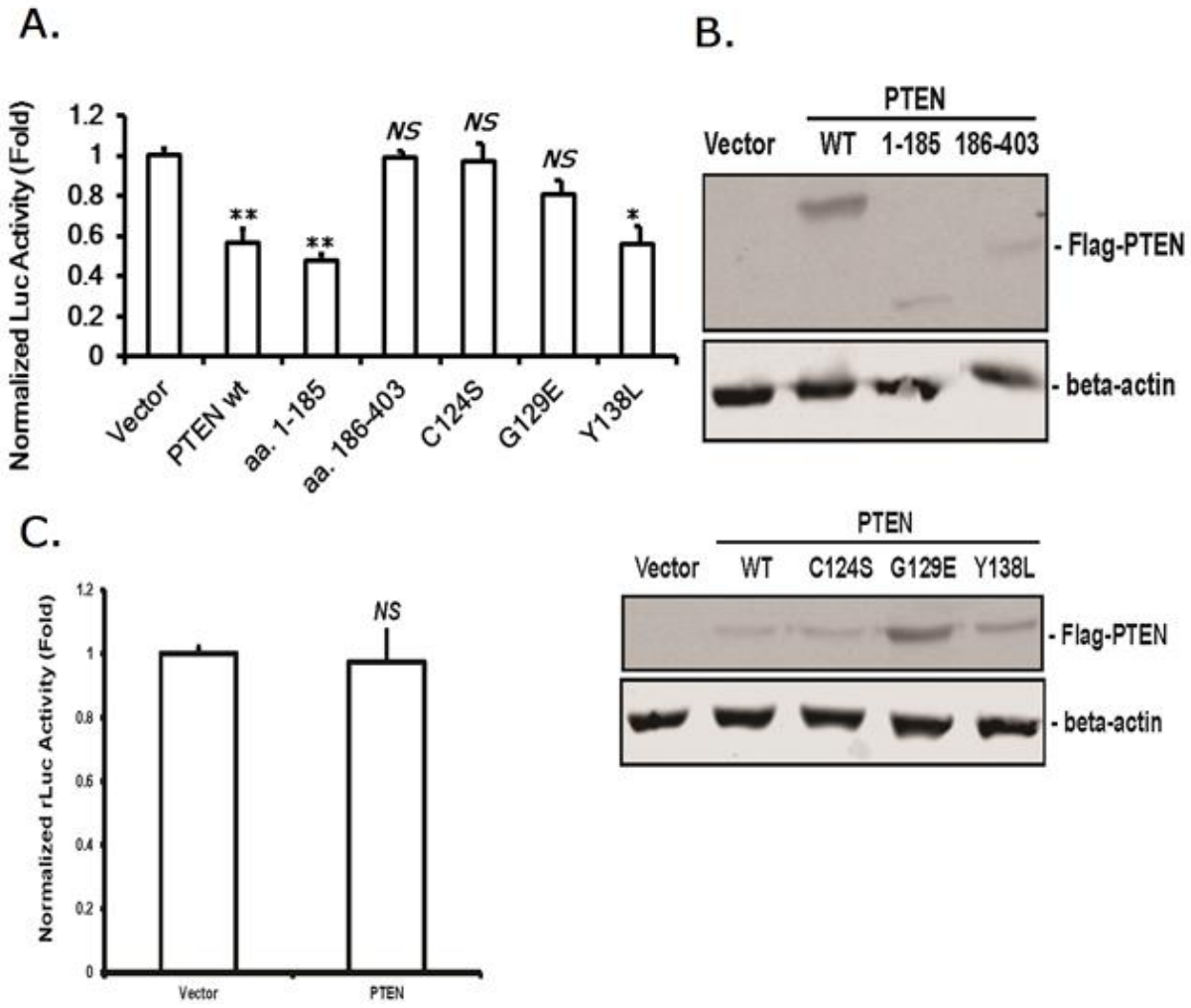

Fig. 9.3. PTEN protein phosphatase activity is required for inhibition of HCV replication in HCV genomic replicon cells. (A) Huh-7 cells with an HCV-2a J6/JFH-1 (p7-rLuc-2A) genomic replicon were transfected with plasmids expressing wild-type Flag-PTEN (PTEN WT), truncated mutants, phosphatase deficient mutants, or empty vector. Forty eight hours after transfection, luciferase assay was performed using the cell lysates. This experiment was performed in triplicate. (B). The protein levels of PTEN and β-actin were determined by Western blotting using anti-PTEN and anti-β-actin antibodies, respectively. (C). Huh-7 cells with an HCV-2a JFH-1 rLuc subgenomic replicon were transfected with plasmid vector or PTEN expressing plasmid. This experiment was performed in triplicate. Luciferase assay was performed 48 hours after transfection. Luciferase activities were expressed as fold changes relative to vector control; ** $p \leq 0.01$, *** $p \leq 0.001$, or *NS* for not significant.

9.5.5 PTEN protein phosphatase activity is required for inhibition of HCV secretion

Data presented so far indicate various effects of PTEN on HCV entry, replication, and translation. A previous study found no effect of PTEN on HCV replication, but identified an inhibition of HCV secretion by PTEN [259]. Given the apparent discrepancy, we felt necessary to examine whether PTEN regulates HCV secretion in our hands. To this end, Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were transfected with vector or plasmids expressing PTEN, wild-type or phosphatase mutants, as per the publication that reported the effect of PTEN on HCV secretion [259]. Virus-containing supernatants were used to infect Huh-7.5 cells and the luciferase activity measured. Because we found negative effects of PTEN on HCV RNA replication, it thus becomes necessary to normalize the luciferase activities measured against the corresponding HCV RNA levels in the initial Huh-7.5 cells as an indication of HCV secretion. As shown in Fig. 9.4E, overexpression of PTEN significantly reduced HCV secretion. To determine if any of the phosphatase activities of PTEN were required for this effect, we once again used phosphatase deficient mutants of PTEN. The results showed that the lipid phosphatase deficient mutant G129E inhibited HCV secretion, whereas the protein phosphatase deficient Y138L and the lipid and protein phosphatase deficient C124S mutants had no effect on HCV secretion (Fig. 9.4E). These data indicate that PTEN protein phosphatase activity is required for inhibiting HCV secretion. Interestingly, this finding is consistent with the previous study just mentioned [259].

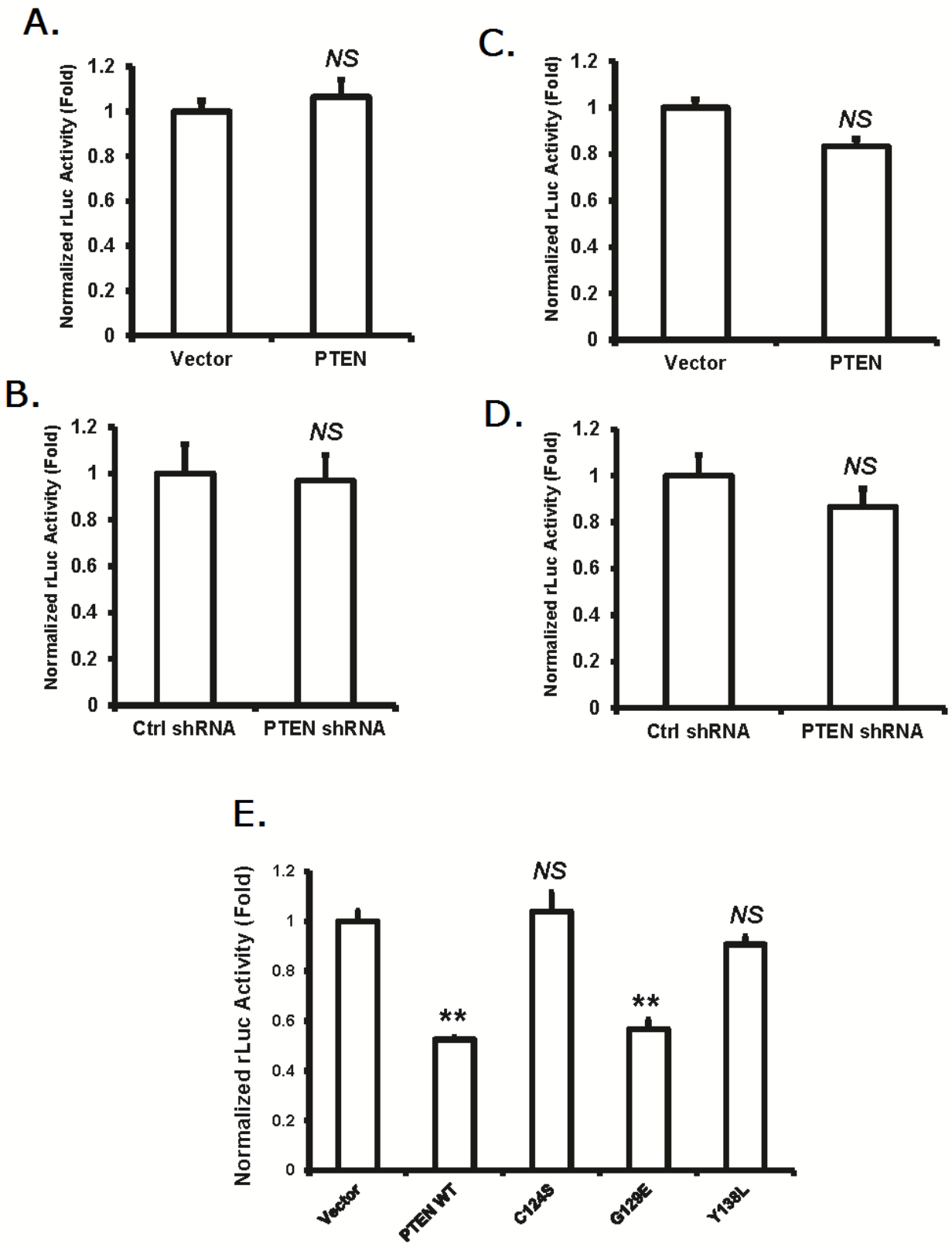
A.
Normalized rLuc Activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
NS
Vector
PTEN
C.
Normalized rLuc Activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
NS
Vector
PTEN
B.
Normalized rLuc Activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
NS
Ctrl shRNA
PTEN shRNA
D.
Normalized rLuc Activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
NS
Ctrl shRNA
PTEN shRNA
E.
Normalized rLuc Activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
**
NS
**
NS
Vector
PTEN WT
C124S
G129E
Y138L

Fig. 9.4. PTEN has no effect on HCV translation, but inhibits HCV secretion. (A and C). Huh-7 cells were co-transfected with a monocistronic HCV-2a translation rLuc reporter RNA (A) or a full-length HCV-2a J6/JFH-1 (p7-rLuc-2A) GNN RNA (C), and plasmid vector or PTEN-expressing plasmid. Luciferase assay was performed 24 hours after transfection. (B and D). Huh-7.5 cells expressing inducible PTEN shRNA or non-silencing control shRNA were treated with 1 μg/mL of Doxycycline for 48 hours. Then the cells were transfected with a monocistronic HCV-2a translation rLuc reporter RNA (B) or a full-length HCV-2a J6/JFH-1 (p7-rLuc-2A) GNN RNA (D). Luciferase assay was performed six hours after transfection. (E). Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were transfected with plasmid vector, or plasmids expressing PTEN, wild-type or phosphatase deficient mutants. Forty eight hours after transfection, (i) the HCV RNA levels in these "donor" cells were determined by luciferase assay, and (ii) the supernatants were collected to infect Huh-7.5 cells. Luciferase assay was performed 72 hpi and normalized against those in the corresponding "donor" cells. The statistical differences between samples were demonstrated as ** if $p \leq 0.01$, or *NS* for not significant. All experiments were performed in triplicate.

9.5.6 PTEN interacts with HCV core

Our results indicated a role of HCV structural proteins in viral replication inhibition by PTEN (Fig. 9.3). Among the three HCV structural proteins, the core protein is more likely to play a role in regulating viral replication than the two envelope proteins. In addition, both PTEN and HCV core proteins often exert their functions through protein-protein interactions [271-273]. Therefore, we investigated whether PTEN interacts with the core protein. Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were transfected with plasmids expressing GST-PTEN or GST and GST-pull down assay was performed. As shown in Fig. 9.5A, HCV core protein was present in the pulled down complex by GST-PTEN, but not GST itself, suggesting an association between these two proteins. To determine the key regions of PTEN responsible, we used truncated PTEN in the GST pull-down assay. Results showed that PTEN aa. 1-186, but not aa. 187-403, was able to pull down the HCV core protein (Fig. 9.5A). We also determined whether the phosphatase deficient mutants of PTEN could pull down HCV core. Fig. 9.5A showed that the C124S, G129E, and Y138L mutants were able to pull down HCV core protein, suggesting these amino acid residues were not involved. These data indicate that the PTEN aa. 1-186 fragment is essential for the association with HCV core protein.

To determine whether PTEN directly interacts with HCV core, His_6-HCV core and GST-PTEN proteins were purified from *E. coli* and used in the GST pull-down assay. Purified His_6-BFP and GST proteins were used as the control proteins. Fig. 9.5B showed that His_6-HCV core was pulled down by GST-PTEN, but not by GST itself. No His_6-BFP was detected in the pull down complexes by GST or GST-PTEN. These data indicate that PTEN directly interacts with HCV core protein.

9.5.7 HCV core R50 in domain I is the key residue required for the interaction with PTEN

Next, we determined the regions and residues of HCV core for the interaction with PTEN. HCV core contains two domains, domain I and domain II [274]. To determine which domain of HCV core was required for the interaction with PTEN, Huh-7 cells were co-transfected with plasmids expressing GST-PTEN, and Flag-tagged HCV core fused with EGFP. Results of the GST pull down assays showed that full-length core as well as domain I interacted with PTEN,

whereas domain II did not (Fig. 9.5C). No interactions were detected between proteins involving control proteins EGFP and GST (Fig. 9.5C and D). These data suggest that the domain I of HCV core is required for the interaction with PTEN.

To map the interacting region within domain I (aa. 1-118), we constructed plasmids expressing truncated domain I and used them in GST pull down assays. We observed that PTEN interacted with HCV core Δaa. 1-41, but not Δaa. 1-59 (Fig. 9.6A). These data indicated that HCV core aa. 42-59 contains the residues for interaction with PTEN. To identify the residue(s) responsible for interaction with PTEN, we employed the alanine scanning approach by mutating groups of two or three amino acid residues at a time into alanines. We found that the P42AR43AL44A and T49AR50A mutants did not interact with PTEN, whereas the G45AV46AR47A, K51AT52AS53A, E54AR55AS56A, and Q57AP58AR59A mutants could still be pulled down by GST-PTEN (Fig. 9.6B). These data suggested that the amino acid residues P42, R43, L44, T49, or R50 of HCV core are likely involved in the interaction with PTEN. We therefore substituted these residues with alanines, one by one, and performed GST pull down assays. PTEN interacted with the P42A and R43A mutants, but not the L44A, T49A or R50A mutants (Fig. 9.6C). These data indicated that, when aa. 1-41 is deleted, the amino acid residues L44, T49 and R50 of HCV core are involved in the interaction with PTEN.
Because the aa. 1-41 of HCV core contains a helix-loop-helix structure as per molecular modeling [275], deletion of this structure may have an impact on the overall conformation of the HCV core protein and thus affect protein-protein interactions. We therefore tested each of the L44A, T49A and R50A mutations in the context of full-length HCV core and determined the interaction with PTEN. GST pull-down assay results showed that the full-length L44A and T49A mutant core proteins could still interact with PTEN, whereas the R50A mutation abolished the interaction completely (Fig. 9.6D). Taken together, these data indicated that R50 of HCV core domain I is the key residue responsible for the interaction with PTEN.

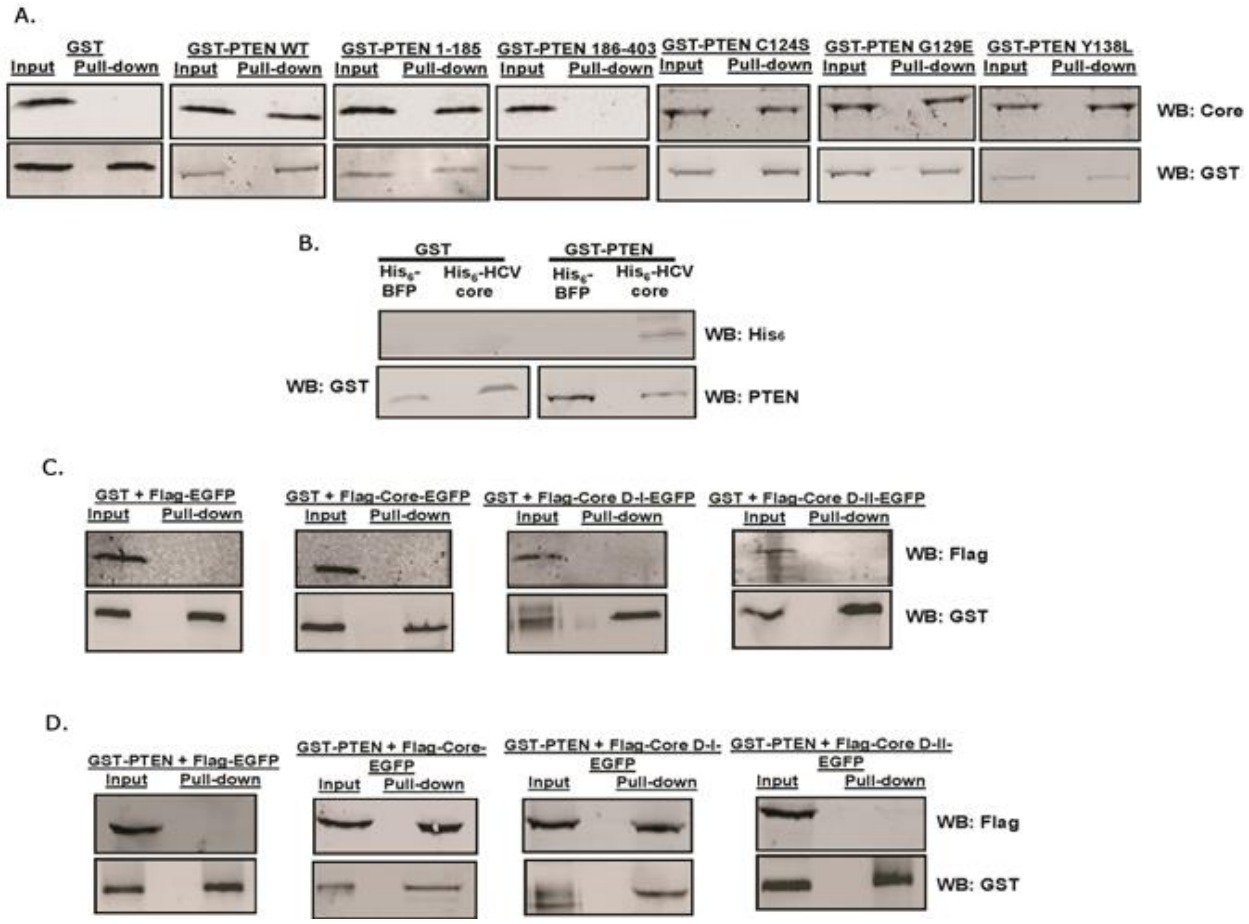

Fig. 9.5. PTEN interacts with the domain I of HCV core. (A). Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A) replicon were transfected with plasmids expressing GST, GST-PTEN wild-type or GST-PTEN mutants. At 48 hours after transfection, cell lysates were subjected to GST pull-down assay. Input and pull-down products were analyzed by Western blotting (WB) using anti-HCV core and anti-GST antibodies, respectively. (B). Purified His_6-BFP (blue fluorescent protein), His_6-HCV core, GST and GST-PTEN proteins were subjected to GST pull-down assay. Pull-down products were analyzed by Western blotting (WB) using anti-His_6, anti-GST and anti-PTEN antibodies, respectively. (C and D). Huh-7 cells were co-transfected with plasmids expressing Flag-tagged HCV core, either full-length, domain I (D-I; aa. 1-118), or domain II (D-II, aa. 119-178), together with plasmids expressing GST (C) or GST-PTEN (D). The core proteins were expressed as fusion proteins with EGFP (enhanced green fluorescent protein). Flag-tagged EGFP-expressing plasmid was included as negative control. At 48 hours after transfection, cell lysates were subjected to GST pull-down assay. Input and pull-down products were analyzed by Western blotting (WB) using anti-Flag and anti-GST antibodies, respectively.

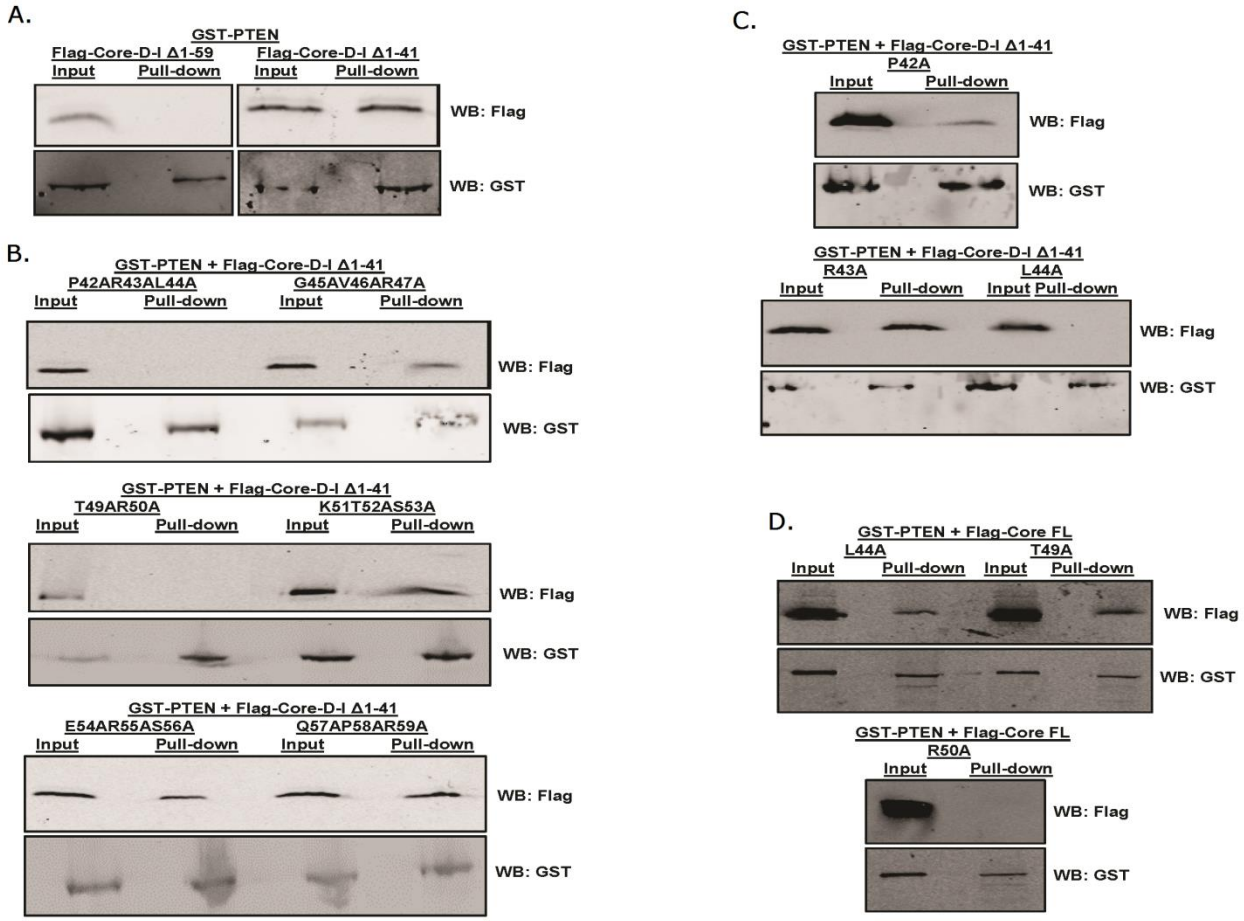

Fig. 9.6. HCV core R50 is the key residue for interaction with PTEN. Huh-7 cells were co-transfected with GST-PTEN-expressing plasmid, together with plasmids expressing truncated HCV core, either wild-type (A), with multiple (B) or single (C) amino acid mutations, or full-length (FL) HCV core with single amino acid mutations (D). The core proteins have a Flag-tag at the N-termini. At 48 hours after transfection, cell lysates were subjected to GST pull-down assay. Input and pull-down products were analyzed by Western blotting (WB) using anti-Flag and anti-GST antibodies, respectively.

9.5.8 HCV core interacts with endogenous PTEN through R50

Data generated so far have shown an interaction between HCV core and PTEN after ectopic expression. It is necessary to demonstrate whether HCV core interacts with endogenous PTEN in HCV replicating cells. Therefore, we generated Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) replicating RNA. The addition of a Flag tag to the core sequence does not have measurable effects on HCV replication [264] and allows convenient co-immunoprecipitation (co-IP) using a Flag-specific antibody. As shown in Fig. 9.7A, PTEN was present in the immunoprecipitates with anti-Flag antibody, suggesting that HCV core interacts with the endogenous PTEN protein in HCV replicating cells. To determine the role of R50 of HCV core in this interaction, we also generated Huh-7 cells harboring HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) replicating RNA. PTEN protein was not detected in the immunoprecipitates using the R50A lysates (Fig. 9.7A). These data demonstrated that HCV core R50 is the key residue for the interaction with endogenous PTEN in HCV replicating cells.

9.5.9 The interaction between PTEN and HCV core is required for inhibiting HCV replication by PTEN

To determine whether PTEN-HCV core interaction plays a role in regulating HCV replication, HCV RNA levels were quantified in wild-type and R50A HCV replicating cells after overexpression of PTEN. We observed that overexpression of PTEN inhibited HCV replication in wild-type, but not in R50A, HCV replicating cells (Fig. 9.7B and C), suggesting a role of PTEN - HCV core interaction. To study whether the interaction between PTEN and HCV core can regulate the PTEN level as a mechanism of modulating HCV RNA replication, we determined the amounts of PTEN in the HCV RNA replicating cells by Western blotting. As shown in Fig. 9.7D, the PTEN protein levels in Huh-7 cells harboring wild-type replicating HCV RNA (HCV J6/JFH-1 (p7-rLuc-2A) and HCV J6 core-Flag/JFH-1 (p7-rLuc-2A)) were lower than those in Huh-7 cells harboring HCV R50A replicating RNA (HCV J6 core R50A-Flag/JFH-1 (p7-rLuc-2A)) and in the parental Huh-7 cells. These results suggested that the PTEN level is reduced in HCV replicating cells most likely due to its interaction with HCV core.

9.5.10 HCV core domain I increases the lipid phosphatase activity of PTEN

The data presented so far have demonstrated that the phosphatase activities of PTEN are required for its inhibitory effects on HCV. In addition, we showed that the interaction between HCV core and PTEN is involved in inhibiting HCV replication by PTEN. Because the phosphatase activities of PTEN can be modulated upon interacting with a protein [51], we wanted to determine whether the HCV core - PTEN interaction has an effect on the phosphatase activities of PTEN. We employed an enzymatic malachite green assay to measure the lipid phosphatase activity of PTEN after incubating with HCV core protein. Because we encountered some difficulty in obtaining the full-length core protein in the quantity and purity required by the assay, we instead used HCV core domain I protein, the interacting domain with PTEN. To determine the effect of the HCV core - PTEN interaction on PTEN's phosphatase activities, we also used HCV core domain I R50A mutant protein in the phosphatase assay. The lipid phosphatase activity of PTEN at 20 nM was measured after incubating with increasing amounts of HCV core domain I protein, either wild-type or with the R50A mutation. As shown in Fig. 9.7E, in the presence of the wild-type HCV core domain I protein at the three lowest concentrations tested, there was a steady increase in the lipid phosphatase activity of PTEN, whereas no activity was measurable in the presence of the R50A protein. With increasing amounts of the core domain I proteins, the lipid phosphatase activity of PTEN also increased. Except for the highest concentration tested, incubation with the wild-type core protein resulted in significantly higher lipid phosphatase activity of PTEN than the R50A protein. These data indicated that the presence of the HCV core domain I protein increases the lipid phosphatase activity of PTEN with the wild-type core being more efficient than the R50A core.

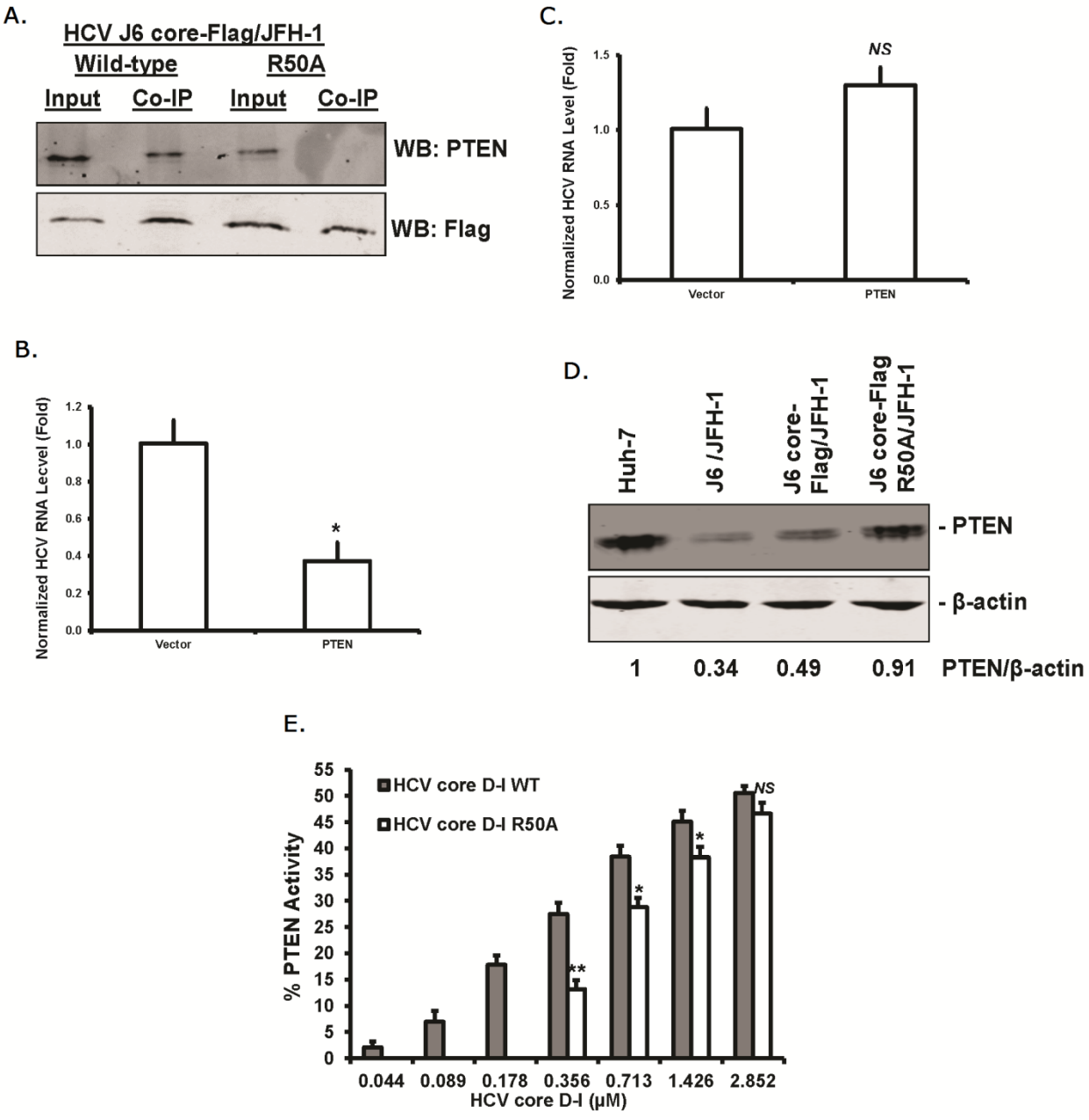
A.
HCV J6 core-Flag/JFH-1
Wild-type
R50A
Input
Co-IP
Input
Co-IP
WB: PTEN
WB: Flag
B.
Normalized HCV RNA Lecvel (Fold)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Vector
PTEN
C.
Normalized HCV RNA Level (Fold)
1.5
1.0
0.5
0.0
NS
Vector
PTEN
D.
Huh-7
J6 /JFH-1
J6 core-Flag/JFH-1
J6 core-Flag R50A/JFH-1
- PTEN
- β-actin
1
0.34
0.49
0.91
PTEN/β-actin
E.
% PTEN Activity
HCV core D-I WT
HCV core D-I R50A
55
50
45
40
35
30
25
20
15
10
5
0
NS
*
*
**
0.044
0.089
0.178
0.356
0.713
1.426
2.852
HCV core D-I (μM)

Fig. 9.7. PTEN-HCV core interaction inhibits HCV replication and increases lipid phosphatase activity of PTEN. (A). The lysates prepared from Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) or HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) replicating RNAs were subjected to immunoprecipitation (IP) using an anti-Flag antibody. Input and IP products were analyzed by Western blotting (WB) using anti-PTEN and anti-HCV core antibodies, respectively. This experiment was performed in duplicate. (B and C). Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (B) or HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) (C) replicating RNAs were transfected with vector or PTEN-expressing plasmid. At 48 hours after transfection, HCV RNA level was determined by reverse transcription real-time PCR using HCV-2a-specific primers. The transcript level of β-glucuronidase (GUSB) was used for normalization. The statistical differences between samples were demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, or *NS* for not significant. This experiment was performed in duplicate. (D). The protein level of PTEN in parental Huh-7 cells, Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A), HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) or HCV-2a J6 core-Flag R50A /JFH-1 (p7-rLuc-2A) was determined by Western blotting using anti-PTEN antibody. The level of β-actin was also determined using anti-β-actin antibody. Relative PTEN band intensities against β-actin were given underneath each sample. This experiment was performed in triplicate. (E). Purified PTEN protein (20 nM) was incubated with increasing amounts of HCV core domain I (HCV core D-I) proteins, wild-type or R50A, as indicated. The phosphatase activity of PTEN towards PtdIns(3,4,5)P_3 was determined in the presence of increasing concentrations of the HCV core D-I proteins and presented relative to PTEN alone (set to a value of 1). The statistical differences between samples were demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, or *NS* for not significant. This experiment was performed in triplicate.

9.6 Discussion

Although the effects of the PI3K/Akt signaling pathway on the HCV life cycle are relatively well documented [255-258], the role of PTEN is less well defined. In this study, we showed that PTEN inhibited HCV entry, replication and secretion through its different PTEN phosphatase activities. Furthermore, we showed that HCV core interacts with PTEN, regulates the steady level and lipid phosphatase activity of PTEN.

We first determined the HCV RNA levels after HCV infection upon ectopic expression of PTEN protein. We showed an approximately 50% reduction in HCV RNA levels after PTEN overexpression with no measurable effects on cell viability (Fig. 9.1). Moreover, the regulation of HCV RNA was confirmed by decreasing PTEN levels by PTEN-specific shRNA. More importantly, the inhibitory effects of PTEN on HCV RNA levels were dose-dependent and PTEN-specific (Fig. 9.1). Because PTEN can potentially regulate different steps of the HCV life cycle, we next studied the effects of PTEN on HCV entry, replication, and translation.

HCV entry occurs through viral interaction with numerous cellular receptors [247]. A recent study has demonstrated that the PI3K/Akt signaling pathway stimulates HCV entry [256]. Because one of the major functions of PTEN is to supress the PI3K/Akt signaling pathway [45], one would expect an inhibitory effect of PTEN on HCV entry. Indeed we showed that overexpression of PTEN reduced, whereas knocking down PTEN enhanced, HCV entry (Fig. 9.2). Furthermore, we showed that the lipid phosphatase activity of PTEN is involved in inhibiting HCV entry (Fig. 9.2).

We next studied the effect of PTEN on HCV replication in HCV replicon cells to bypass the entry step. Interestingly, we found that PTEN inhibited the replication of HCV genomic RNA, but not subgenomic RNA (Fig. 9.3). These results suggested a role of viral structural proteins in replication inhibition by PTEN. A previous study found that overexpression of PTEN does not have a significant effect on HCV replication using an HCV Jc1 virus [259]. The reasons for the discrepancy are not clear and whether it is due to different experimental conditions or viral strains should be investigated.

HCV RNA translation is primarily mediated by the internal ribosomal entry site within the viral 5'-UTR with viral and cellular factors playing a regulatory role [248]. We recently demonstrated that the PI3K/Akt signaling pathway positively regulates HCV RNA translation

[258]. Interestingly however, no effect of PTEN on HCV RNA translation was observed in the current study (Fig. 9.4). This is not entirely unexpected because there exist examples where the phenotypes of PTEN (down-regulation) may not always result in altered Akt (up-regulation) [45,276]. Our results imply that the PI3K/Akt and PTEN modulate HCV RNA translation through different mechanisms, which warrant further investigation.

To understand how PTEN inhibits HCV genomic RNA replication, but not subgenomic RNA replication, we sought to determine whether PTEN interacts with the HCV core proteins as we reasoned that the core protein will more likely to be involved than E1 and E2 proteins. We indeed found that PTEN interacted directly with HCV core protein (Fig. 9.5). Furthermore, we identified the arginine residue at position 50 (R50) within domain I of HCV core protein as the key residue required for mediating the interaction (Fig. 9.6). More importantly, when R50 was mutated, PTEN could no longer inhibit HCV replication (Fig. 9.7), suggesting a functional significance of this interaction. Previous studies have shown that the R50 residue of HCV core protein is involved in interacting with the NS5A protein and is required for HCV infectivity [264,277]. As an additional function of this residue, our results have demonstrated that R50 has a role in regulating HCV replication by interacting with PTEN.

Previous studies have demonstrated that the expression of HCV core results in decreased levels of PTEN protein through either blocking PTEN mRNA translation [147] or activation of NF-κB [149]. Therefore we were interested in determining the role of HCV core - PTEN interaction in modulating PTEN levels. We found that the replication of wild-type HCV was associated with decreased PTEN protein level, which was restored to a significant extent when R50 was mutated into alanine (Fig. 9.7). These data indicate that HCV core decreases the steady level of PTEN through protein - protein interaction as yet another mechanism.

Protein - protein interactions play an important role in modulating the phosphatase activities [51]. We showed that the phosphatase activities of PTEN were involved in regulating different steps in the HCV life cycle (Fig. 9.1, 9.2 and 9.3). These data prompted us to determine whether the HCV core - PTEN interaction has an effect on the phosphatase activities of PTEN in an *in vitro* enzymatic assay. We found that the presence of HCV core domain I protein increased the lipid phosphatase activity of PTEN in a dose-dependent manner (Fig. 9.7). In contrast, the HCV core domain I R50A mutant protein was only able to enhance the lipid phosphatase activity

at high concentrations tested (Fig. 9.7). These results strongly suggest that PTEN - HCV core interaction plays a role in the regulation of PTEN activities.

In summary, we presented evidence to support an inhibitory role of PTEN in regulating HCV entry, replication and secretion. This suggests that liver cancers with reduced expression of PTEN could be more susceptible to HCV infection [278]. Mechanistically, we demonstrated that HCV core interacts with PTEN and may modulate the functions of PTEN through multiple mechanisms. Our results should provide an additional avenue for developing therapeutics for HCV infection and the resultant hepatocellular carcinoma.

9.7 Acknowledgements

We would like to thank Dr. Charles Rice for providing Huh-7.5 cells and HCV-2a J6/JFH-1 p7-rLuc-2A plasmid, Dr. Takaji Wakita for providing HCV-2a JFH-1 plasmid. We thank Katie Lin for her contribution in the early stage of the project. This work was supported by grants from Canadian Institutes of Health Research (CIHR), Saskatchewan Health Research Foundation, and Natural Sciences and Engineering Research Council of Canada to QL. This work was also supported by a grant from the Saskatchewan Cancer Agency to DHA. The research leading to these results has also received funding from CIHR (FRN# NHC-142832) and the Public Health Agency of Canada (PHAC) in the form of a Ph.D. scholarship to QW. This article is published with the permission of the Director of VIDO-InterVac, journal series no. 782.

10.0 THE ROLE OF PTEN-LONG ON HCV REPLICATION

In the previous chapter, we showed that PTEN inhibits HCV entry, replication and secretion. HCV core interacts with PTEN and inhibits PTEN expression, which enhances HCV replication. PTEN has an isoform called PTEN-Long which can export into extracellular compartments, enter neighboring cells, and induce signaling events in recipient cells. In the following chapter, we set to determine if PTEN-Long affects HCV replication and if HCV core interacts with PTEN-Long.

11.0 TREATMENT WITH PTEN-LONG PROTEIN INHIBITS HEPATITIS C VIRUS REPLICATION

Qi Wu[1] and Qiang Liu[2,*]

[1]VIDO-InterVac and Department of Veterinary Microbiology, University of Saskatchewan,

[2]VIDO-InterVac, Department of Veterinary Microbiology and School of Public Health,

University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Running title: PTEN-Long inhibits HCV replication

Keywords: Hepatitis C virus; replication; PTEN-Long; core

* Corresponding author.

VIDO-InterVac

University of Saskatchewan,

120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.

Fax: +1 306 966 7478.

E-mail address: qiang.liu@usask.ca. (Q. Liu).

11.1 Authors' contribution

All the experiments within this chapter were performed by Qi Wu. The manuscript was written by Qi Wu and edited by Qiang Liu.

11.2 Abstract

Hepatitis C virus (HCV) infection is a confirmed risk factor for hepatocellular carcinoma (HCC). Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) possesses tumor suppression function that is frequently defective in HCC tumors. PTEN-Long, an isoform of PTEN, functions in a cell non-autonomous manner. In this study, we demonstrated that intracellular overexpression of PTEN-Long inhibits HCV replication. More importantly, we showed that treatment with extracellular PTEN-Long protein inhibits HCV replication in a dose dependent manner. Furthermore, we showed that PTEN-Long interacts with HCV core protein and this interaction is required for HCV replication inhibition by PTEN-Long. In summary, we demonstrated, for the first time, that PTEN-Long protein, an isoform of the canonical PTEN and in the form of an extracellular protein treatment, inhibits HCV replication. Our study offers an opportunity for developing additional anti-HCV agents.

11.3 Introduction

Despite the approval of anti-viral drugs, hepatitis C virus (HCV) infection continues to be a significant public health problem with hepatocellular carcinoma (HCC) as the most deadly clinical outcome [246]. HCV has a single-stranded RNA genome encoding a polyprotein that is processed by cellular and viral proteases to generate structural and non-structural proteins [247]. Although it is a structural protein, HCV core has been reported to possess many regulatory functions including tumorigenesis [143,249].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a dual phosphatase with lipid and protein phosphatase activities [45,250]. These phosphatase activities render PTEN to function as a tumor suppressor [45]. PTEN has been characterized as one of the

most frequently mutated or deleted genes in various tumors including HCC [45,49]. For instance, three single amino acid mutations in the phosphatase domain have been identified: C124S (lipid and protein phosphatase defective), G129E (lipid phosphatase activity defective), and Y138L (protein phosphatase defective) [50]. As a protein product translated from an alternative start codon 519 bp upstream of the ATG initiation sequence of PTEN, a longer isoform of PTEN, termed PTEN-Long, was discovered very recently [59,61,279]. PTEN and PTEN-Long possess comparable phosphatase activities [59]. PTEN-Long has 173 N-terminal extra amino acids with a poly-arginine region. This region enables PTEN-Long to be exported into extracellular compartments, enter neighboring cells, and induce signaling events in recipient cells [59].

We previously demonstrated that PTEN can inhibit HCV replication through interacting with HCV core protein (Chapter 9). Whether PTEN-Long affects HCV replication has not been studied. In this study, we showed that intracellular expression of or extracellular treatment with PTEN-Long protein can inhibit HCV replication. Furthermore, we showed that PTEN-Long HCV core protein interaction is involved in the inhibitory effect of PTEN-Long on HCV replication.

11.4 Materials and Methods

11.4.1 Plasmid constructs

Plasmid expressing Flag-tagged PTEN was described previously (Chapter 9). Plasmids expressing PTEN-Long-V5-His_6 (Addgene plasmid #49417) and PTEN-V5-His_6 (Addgene plasmid #49420) in the bacterial JpExpress404 vector or in the pcDNA3 vector were obtained from Addgene or Dr. Ramon Parsons [59]. Bacterial or eukaryotic expression plasmids encoding RFP (red fluorescent protein) fusion proteins with PTEN or PTEN-Long were constructed as described [59]. Plasmids expressing PTEN-Long mutants with single amino acid substitutions C297S, G302E, or Y311L were generated in reference to the corresponding mutations of PTEN [259]. His_6-BFP (blue fluorescent protein)-expressing plasmid in the pT7 His_6-SUMO vector (Lucigen) was described previously [268]. All plasmids were constructed using standard methods and confirmed by DNA sequencing.

11.4.2 Cell culture and transfection

Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Huh-7 cells with replicating HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) or HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) RNAs were maintained with culture medium plus G418 (Enzo Life Sciences) as described previously (Chapter 9). Cells were transfected with plasmid DNA using the calcium phosphate precipitation method [163] or the Jet-PEI transfection reagent (Polyplus Transfection) [258].

11.4.3 Luciferase assay

Cells were lysed in Passive Lysis Buffer (Promega) and the firefly or renilla luciferase activities were measured by Luciferase Assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Firefly or renilla luciferase levels were normalized to the protein concentrations determined by the Bradford assay (Bio-Rad Laboratories). These assays were performed at least three times and the results analyzed for statistical differences by the Student's *t* test. A p value of ≤ 0.05 was considered statistically significant.

11.4.4 Co-immunoprecipitation (Co-IP) and Western blotting

Co-IP and Western blotting were performed as described previously (Chapter 9). For Flag co-IP experiments, cells were harvested by radio immunoprecipitation assay (RIPA) buffer and incubated with anti-Flag (Sigma-Aldrich) antibody at 4 ℃ overnight and then incubated with Protein G Sepharose (GE Healthcare) at 4 ℃ for 4 hours. The mixtures were centrifuged at 10,000 rpm for 10 minutes and the supernatants were removed. The pellets were resuspended in SDS lysis buffer and boiled for 10 minutes to elute the proteins. For Western blotting, proteins were subjected to SDS-PAGE and then blotted onto nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBS and incubated with a primary antibody overnight at 4 ℃. Membranes were washed and incubated with a secondary antibody for 1 hour at room

temperature. After a wash with PBST (PBS+0.1% Tween 20), membranes were scanned using Li-Cor Odyssey scanner (ODY-CLx) and band intensities were determined by Quantity One software (Bio-Rad Laboratories). These primary antibodies were used: HCV core (Anogen), PTEN (Cell Signaling Technology), β-actin (CST), and Flag (Sigma-Aldrich). The secondary antibodies used were IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

11.4.5 Purification of recombinant proteins

The expression of recombinant proteins in *E. coli* was induced by IPTG (Thermo Fisher Scientific). His_6-tagged PTEN, PTEN-Long, and BFP proteins were purified by Ni-NTA agarose (Qiagen) as previously described [266].

11.5 Results

11.5.1 Intracellular PTEN-Long inhibits HCV replication

We previously showed that ectopic expression of PTEN inhibits HCV replication (Chapter 9). Whether PTEN-Long, a longer isoform of PTEN, can also regulate HCV replication has not been studied. To investigate the effect of PTEN-Long on HCV replication, we transfected Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) replicating RNA with vector, plasmids expressing PTEN or PTEN-Long and measured HCV replication by luciferase assay. We also used a set of plasmids expressing fusion proteins with RFP, which will enable subsequent experiments – see below. Consistent with our previous results, overexpression of PTEN significantly inhibited HCV replication in comparison to vector (Fig. 11.1A). Expression of the fusion protein PTEN-RFP also inhibited HCV replication as effectively as PTEN itself, while RFP expression had no effect (Fig. 11.1A). Overexpression of PTEN-Long or PTEN-Long-RFP could reduce HCV replication to the same extent as PTEN (Fig. 11.A). The intracellular expression of these proteins was demonstrated by Western blotting (Fig. 11.1B). These results

indicated that both PTEN and PTEN-Long can inhibit HCV replication when expressed intracellularly.

11.5.2 Extracellular PTEN-Long protein treatment inhibits HCV replication

In comparison to the canonical PTEN, a unique feature of PTEN-Long lies in its ability to enter cells as a secreted protein [59]. We therefore were interested in determining whether PTEN-Long as an extracellular protein regulates HCV replication. To this end, we purified His_6-tagged PTEN and PTEN-Long proteins with or without fusion with RFP as previously described [59]. Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) replicating RNA were treated with increasing amounts of PTEN or PTEN-Long protein and HCV replication quantified. PBS and BFP protein were used as controls. As shown in Fig. 11.2A, while HCV replication was not affected by treatment with PBS, BFP, or PTEN proteins in three escalating doses, HCV replication was reduced upon PTEN-Long protein treatment in a dose-dependent manner. These results indicated that extracellular PTEN-Long protein can inhibit HCV replication.

We previously showed that the lipid phosphatase deficient G129E mutant of PTEN can inhibit HCV replication as effectively as the wild-type PTEN, whereas the protein phosphatase deficient Y138L and the lipid and protein phosphatase deficient C124S mutants have no effect (Chapter 9). Next, we wanted to determine whether this is also the case for PTEN-Long as an extracellular protein. We therefore transferred the phosphatase point mutations of PTEN to the corresponding positions in PTEN-Long and purified these proteins for use in the treatment. Fig. 11.2B showed that while mutations in protein phosphatase activity C297S and Y311L no longer inhibited HCV replication, the lipid phosphatase G302E mutant protein had the same inhibitory effect as the wild-type PTEN-Long. These results indicated that the protein phosphatase activity of PTEN-Long is required for inhibiting HCV replication.

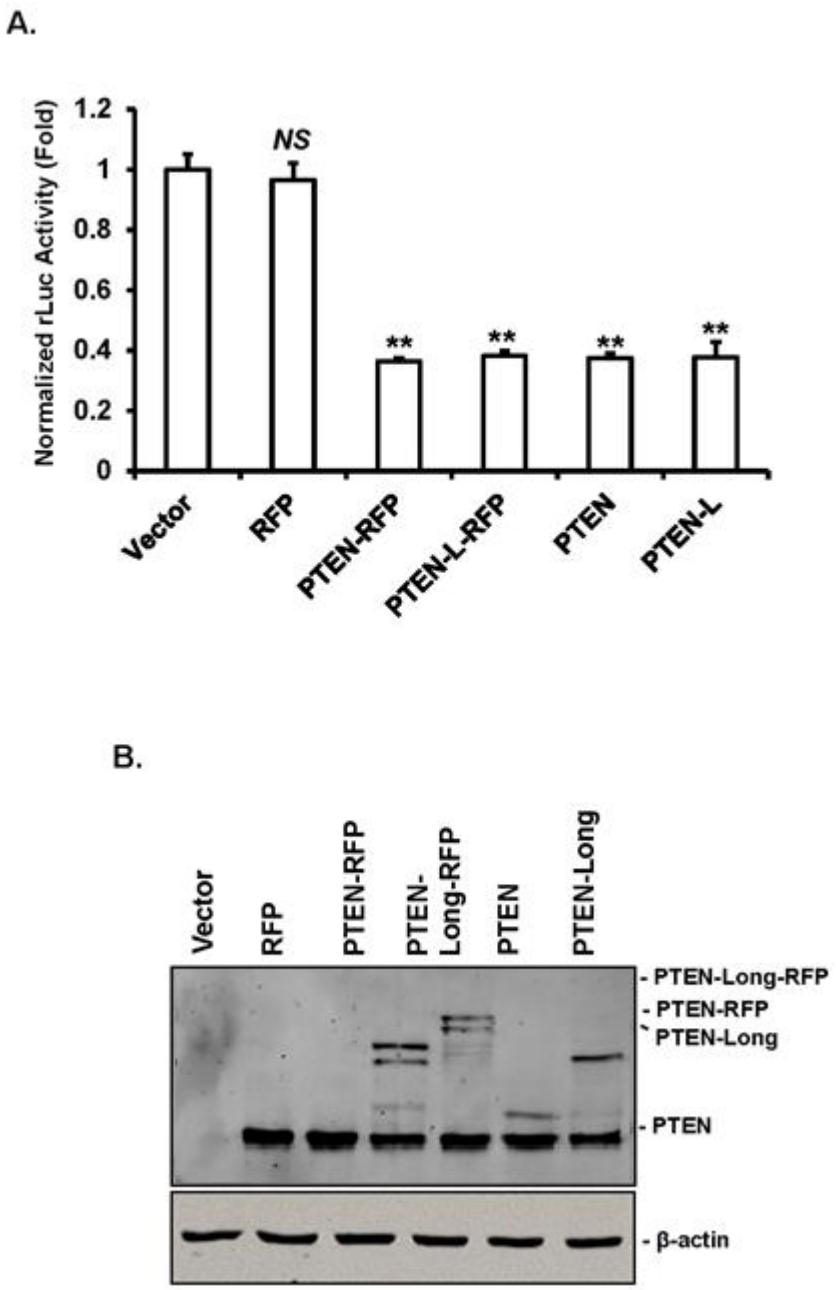

Fig. 11.1. Intracellular expression of PTEN-Long inhibits HCV replication. Huh-7 cells harboring HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were transfected with plasmid vector, or plasmids expressing RFP (red fluorescent protein), PTEN-RFP, PTEN-Long-RFP (PTEN-L-RFP), PTEN, or PTEN-Long (PTEN-L). All proteins have a C-terminal V5-His_6 tag. (A) At 48 hours after transfection, luciferase assay was performed and the statistical differences between samples were demonstrated as ** if $p \leq 0.01$, or *NS* for not significant. This experiment was performed three times. (B) The expression of RFP, PTEN, and PTEN-Long proteins was demonstrated by Western blotting using an anti-PTEN antibody. The levels of β-actin were also determined as loading controls. This experiment was performed three times.

To confirm that PTEN-Long but not PTEN protein can enter the cells, we fused PTEN-Long or PTEN with RFP and detected red fluorescence after treatment. As shown in Fig. 11.2C, red fluorescence was observed after treatment with PTEN-Long-RFP, but not with PTEN-RFP or RFP, indicating that only extracellular PTEN-Long protein can enter the cells. Fusion with RFP did not affect the function of PTEN and PTEN-Long in regulating HCV replication (compare Fig. 11.1D and 11.1A).

Taken together, these results demonstrated that extracellular PTEN-Long protein can enter the cells and inhibit HCV replication.

11.5.3 PTEN-Long inhibits HCV replication through interaction with HCV core

We previously showed that HCV core protein interacts with PTEN through the arginine residue at position 50 (R50) and the interaction is required for the inhibitory effect of PTEN on HCV replication (Chapter 9). To determine whether HCV core also interacts with PTEN-Long and the role of R50, we performed Flag co-immunoprecipitation experiment after transfecting PTEN-Long expressing plasmid into HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) and HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) replicating cells. As shown in Fig. 11.3A, PTEN-Long was detected in the immunoprecipitates using lysates containing wild-type, but not R50A, HCV core. Since PTEN is translated (internally) from the PTEN-Long coding sequence, PTEN protein was also present/absent in the immunoprecipitates (Fig. 11.3A). These results indicated that HCV core interacts with PTEN-Long and requires R50.

Next, we wanted to investigate the role of HCV core - PTEN-Long interaction in regulating HCV replication. HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) replicating Huh-7 cells were treated with 24 nM of BFP, PTEN, PTEN-Long and phosphatase mutant proteins, and PBS before HCV replication measured. As shown in Fig. 11.3B, no effects on HCV replication were observed. These results indicated that PTEN-Long interacts with HCV core, which is required for HCV replication inhibition.

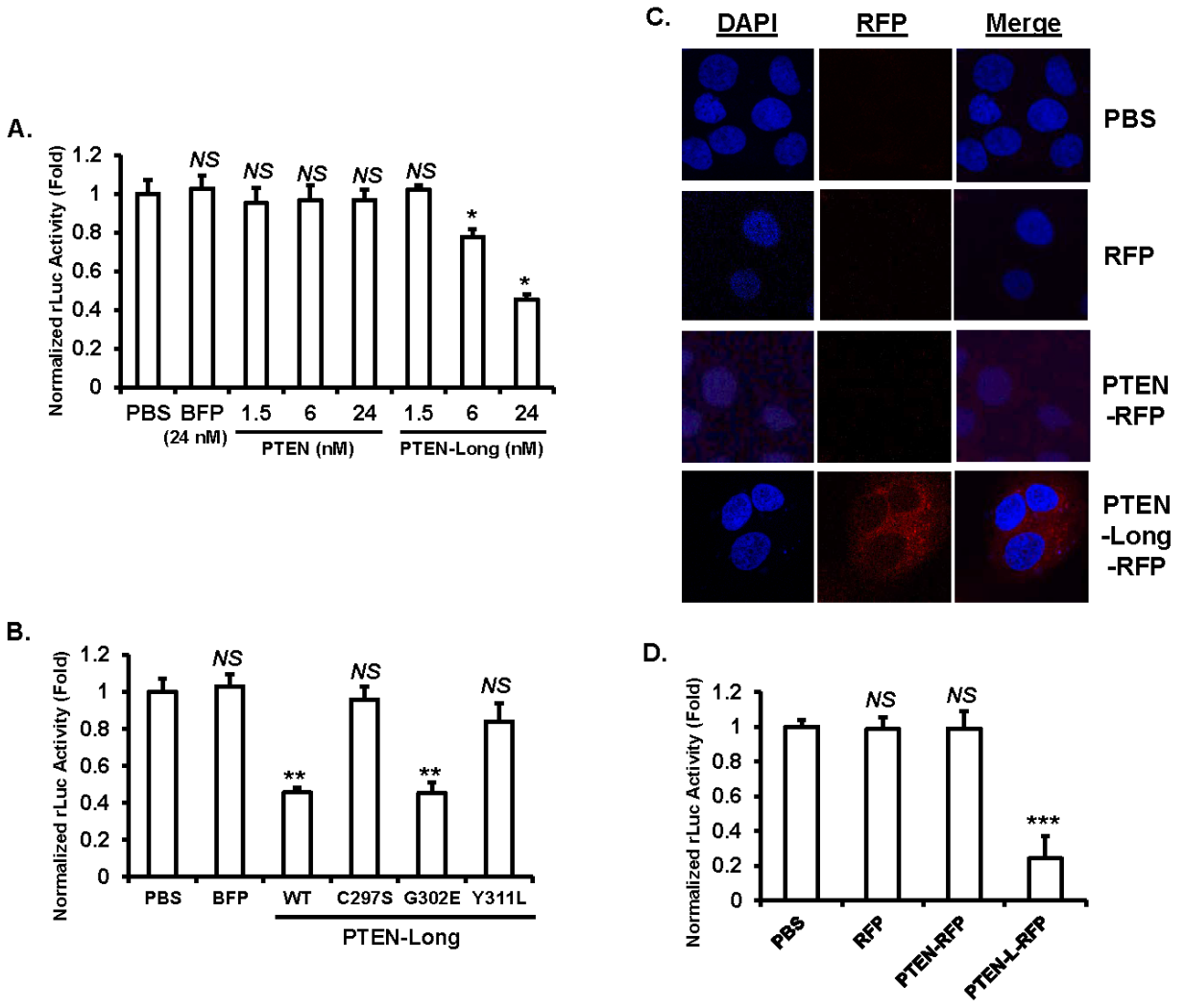

Fig. 11.2. Extracellular treatment with PTEN-Long protein inhibits HCV replication. Huh-7 cells with HCV-2a J6/JFH-1 (p7-rLuc-2A) replicating RNA were treated with BFP, RFP, PTEN, PTEN-Long, PTEN-RFP, or PTEN-Long-RFP proteins or equal volume of PBS (phosphate-buffered saline). All proteins had a His_6-tag and were purified from *E. coli* through affinity chromatography. In (A), 1.5, 6, and 24 nM of PTEN or PTEN-Long proteins and 24 nM of BFP protein were used. In (B, C and D), cells were treated with 24 nM of proteins. At 24 hours after treatment, luciferase assay was performed (A, B and D). The statistical differences between samples were demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$, or *NS* for not significant. These experiments were performed three times. (C) At 6 hours after treatment, cells were stained with DAPI. Images were collected by confocal microscopy and analyzed by the Image J software. This experiment was performed two times.

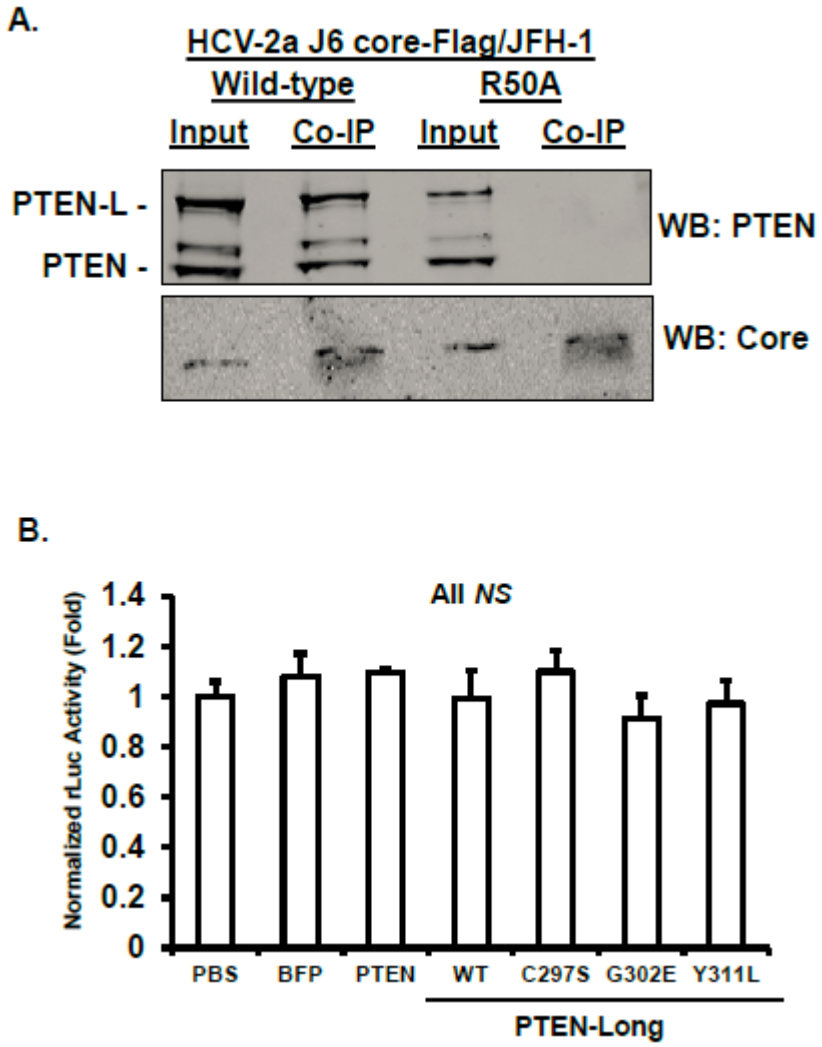

Fig. 11.3. PTEN-Long protein inhibits HCV replication by interacting with HCV core. (A) Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) or HCV-2a J6 core R50A-Flag/JFH-1 (p7-rLuc-2A) replicating RNAs were transfected with plasmid expressing PTEN-Long. Forty eight hours after transfection, cell lysates were subjected to co-immunoprecipitation (co-IP) using an anti-Flag antibody. Input and co-IP products were analyzed by Western blotting (WB) using anti-PTEN and anti-HCV core antibodies, respectively. This experiment was performed three times. (B) Huh-7 cells harboring HCV-2a J6 core R50A-Flag /JFH-1 (p7-rLuc-2A) replicating RNA were treated with 24 nM of BFP, PTEN, PTEN-Long proteins or equal volume of PBS. Luciferase assay was performed at 24 hours after treatment. The statistical differences between samples were demonstrated as *NS* for not significant. This experiment was performed three times.

11.6 Discussion

Viruses are the etiologic agents for more than 10% of tumors in human [280]. However, the mutual interactions between PTEN-Long and oncogenic viruses have yet to be characterized. As an extension to our recent study on the effect of the canonical PTEN on HCV replication (Chapter 9), we characterized the role of PTEN-Long in this study. We showed that intracellular expression of PTEN-Long could inhibit HCV replication as effectively as the PTEN protein (Fig. 11.1). More importantly, we found that HCV replication was inhibited by incubation with purified PTEN-Long protein in a dose-dependent manner (Fig. 11.2). This inhibition is due to the permeability feature of the PTEN-Long protein because we showed that PTEN-Long in the form of an extracellular protein, but not the PTEN protein, can enter HCV replicating cells (Fig. 11.2). As for the mechanisms, we found that the C297S and Y311L mutants of PTEN-Long do not inhibit HCV replication (Fig. 11.2) and PTEN-Long - HCV core protein interaction is required for inhibiting HCV replication by PTEN-Long (Fig. 11.3). Taking our previou study on how PTEN inhibits HCV replication (Chapter 9) into consideration, these results suggest that PTEN-Long, once present inside the cell, exerts its inhibitory effects on HCV replication through similar mechanisms as the canonical PTEN.

In summary, we demonstrated that PTEN-Long inhibits HCV replication. To the best of our knowledge, this is the first report on characterizing the effect of PTEN-Long on a viral replication. The fact that PTEN-Long exerts this inhibitory function in the form of an extracellular protein should provide an additional avenue for developing therapeutics for HCV infection and the resultant hepatocellular carcinoma.

11.7 Acknowledgements

This work was supported by grants from Canadian Institutes of Health Research (CIHR), Saskatchewan Health Research Foundation, and Natural Sciences and Engineering Research Council of Canada to QL. The research leading to these results has also received funding from CIHR (FRN# NHC-142832) and the Public Health Agency of Canada (PHAC) in the form of a Ph.D. scholarship to QW.

12.0 HOW HBV AND HCV REGULATES PTEN AND SREBP-1 AND WHETHER THIS PROCESS ALTERS HBV AND HCV REPLICATION IN HBV HCV CO-INFECTION

In Chapters 3 to 11, we showed that HBx stimulates SREBP-1 activity and enhances HBV replication. We also observed that HCV core inhibits PTEN expression and enhances HCV replication. Loss of PTEN results in activation of the PI3K/Akt pathway which leads to SREBP-1 activation. Previous studies have established that both HBx and HCV core can inhibit PTEN and enhance SREBP-1 activity in HBV or HCV mono-infection. However, the role of HBx and HCV core on the PTEN/SREBP-1 pathway is still not fully understood. On the other hand, whether regulation of the PTEN/SREBP-1 alters HBV and HCV replication in HBV HCV co-infection is not clear. In the following chapter, we set to determine how the PTEN/SREBP-1 pathway affects HBV HCV co-infection.

13.0 THE ROLE OF PTEN AND SREBP-1 IN HBV HCV CO-INFECTION

Qi Wu[1] and Qiang Liu[2,*]

[1]VIDO-InterVac and Department of Veterinary Microbiology, University of Saskatchewan,

[2]VIDO-InterVac, Department of Veterinary Microbiology and School of Public Health,

University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Keywords: Hepatitis B virus; Hepatitis C virus; PTEN; SREBP-1; Co-infection

* Corresponding author.

VIDO-InterVac

University of Saskatchewan,

120 Veterinary Road, Saskatoon, SK, Canada S7N 5E3.

Fax: +1 306 966 7478.

E-mail address: qiang.liu@usask.ca (Q. Liu).

13.1 Authors' contribution

All the experiments within this chapter were performed by Qi Wu. The manuscript was written by Qi Wu and edited by Qiang Liu.

13.2 Abstract

Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infections cause a wide range of liver diseases including hepatocellular carcinoma (HCC). Because these viruses have the same modes of transmission, HBV HCV co-infections are found in approximately seven to twenty million people globally. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), a tumor suppressor, suppresses phosphatidylinositol 3-kinase (PI3K)/Akt pathway and its downstream factor sterol regulatory element binding protein-1 (SREBP-1). In this study, we examined whether HCV affects HBV replication in HCV replicating cells or HBV affects HCV replication in HBV replicating cells. We found that HBV and HCV do not interfere with each other in HCV replicating cells. However, in HBV replicating cells, HBV enhances the early stage of HCV infection. We also showed that HBV replication is regulated by HBx-dependent PTEN/SREBP-1 pathway, while HCV replication is mainly regulated by PTEN knockdown and is also modestly modulated by SREBP-1 knockdown. Moreover, we found that HBx and HCV core do not synergistically inhibit PTEN protein level or enhance SREBP-1 activation. Taken together, this study demonstrated the role of PTEN and SREBP-1 in HBV or HCV mono-infections and HBV HCV co-infection.

13.3 Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections cause severe liver disease worldwide. Globally, approximately 240 to 400 million people are chronically HBV infected and 120 to 200 million people are chronically HCV infected [281]. Because HBV and HCV share the model of transmission, HBV HCV co-infection is common in highly endemic areas. In general, approximately 5%-20% in HBsAg (HBV surface antigen) positive patients are found to be HCV positive and 2%-10% HCV positive patients are found to be HBsAg positive [282]. Compared to HBV or HCV mono-infections, HBV HCV co-infection leads to more severe liver disease and higher incidence of hepatocellular carcinoma (HCC) [283].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase which contains both lipid and protein phosphatase activities. PTEN is considered a tumor suppressor since its lipid phosphatase activity can suppress one of the most critical cancer-promoting pathways, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [45,49]. The activation of Akt inhibits proline-reich Akt substrate 40 kDa (PRAS40) and tuberous sclerosis 1/2 (TSC1/2) and results in the activation of the mammalian target of rapamycin complex 1 (mTORC1) [78]. Activation of mTROC1 stimulates SREBP-1 transcription, processing and nuclear localization [80]. Overall, PTEN negatively regulates SREBP-1 activation through PI3K/Akt/mTORC1 pathway [210].

HBV is a partially double-stranded DNA virus with a genome of 3020-3320 nucleotides (for the full-length strand) and 1700 - 2800 nucleotides (for the short length-strand) [8]. There are four known HBV genes, called C, X, P, and S [9]. The HBV core protein is coded for by gene C. HBeAg is produced by proteolytic processing of the pre-core protein. HBV P proteins are encoded by gene P which functions are important for reconstitution of HBV DNA synthesis. Gene S is the gene that codes for HBsAgs which include large (L), middle (M), and small (S) surface proteins. HBx encoded by gene X is a nonstructural protein which functions as a transcriptional transactivator of host genes [7]. HBx modulates HBV replication through an SREBP-1 dependent manner [284]. Previous studies have demonstrated that HBx inhibits the function of p53 [285] and therefore increases alpha-fetoprotein (AFP) expression [286]. AFP associates with PTEN in the cytoplasm and reduces PTEN level [287].

HCV is a small, enveloped, single positive-stranded RNA virus [23]. The RNA genome of HCV translates into a single large polyprotein which is processed by host and viral proteases to generate ten viral proteins: E1, E2, core, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B. Previous studies have demonstrated that both HCV core and NS5A proteins are involved in the down-regulation of PTEN [147-149]. Previously, we have established that the HCV core interacts with PTEN and HCV core R50A is the key residue of the interaction. The replication of HCV RNA with the R50A mutation is significantly reduced (Chapter 9).

In this study, we showed that HBV can replicate in HCV replicating Huh-7 cells with no mearuable influence on HBV replication by HCV. HCV can also replicate in HBV replicating Huh-7 cells and HBV enhances HCV replication in the early stage of HCV infection. We also showed that PTEN inhibits HBV replication through modulating HBx-dependent SREBP-1 activation. Although HCV replication is enhanced by respective PTEN knockdown or SREBP-1 knockdown, knocking down SREBP-1 does not affect the PTEN-modulated HCV replication. Moreover, we further showed that HBx and HCV core has no synergy in PTEN and SREBP-1.

13.4 Materials and Methods

13.4.1 Plasmid constructs and *in vitro* transcription

The hygromycin resistance gene was inserted into the pcDNA3.1 vector generating plasmid pcDNA3-Hygro^R. Plasmids HBV payw1.2-Luc (Hygro^R) or HBV payw1.2*7-Luc (Hygro^R) were constructed by insertion of a greater-than-unit-length HBV genome (payw 1.2) or a greater-than-unit-length HBV genome without expressing HBx (payw 1.2*7) [219,237] and a firefly luciferase (Luc) gene into the plasmid pcDNA-Hygro^R. Plasmids pFLneo-HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) and pFLneo-HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) were described previously [264] (Chapter 9). Plasmid pFLneo-HCV-2a J6/JFH-1 (p7-rLuc-2A) GNN, the replication-deficient version, was obtained from Dr. Charles Rice [263]. Plasmid pFLneo-HCV-2a J6 core R50A/JFH-1 (p7-rLuc-2A) GNN was constructed by introducing the R50A mutation. We constructed plasmids expressing HBx, HBV precore, HBV L, HBV M or HBV S with a myc-tag at the C-terminus under the control of the elongation factor-1α promoter.

We also constructed plasmid expressing HBV P with a Flag-tag at the N-terminus the control of the elongation factor-1α promoter. Plasmid expressing human PTEN with an N-terminal Flag-tag, non-silencing control shRNA, PTEN shRNA were described previously (Chapter 9). Non-silencing control microRNA (miRNA) and SREBP-1 miRNA were described previously [137,288].

13.4.2 Cell culture, transfection, nuclear fractionation, and generation of stable cell lines

Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS). Cells were transfected with plasmid DNA and/or HCV RNAs using the calcium phosphate precipitation method [163] or the jetPEI transfection reagent (Polyplus Transfection) [288], respectively. Nuclear fractionation was described previously [237]. To establish HBV replicating cells, Huh-7 cells were transfected with HBV genome plasmid DNA and selected by Hygromycin (Enzo Life Sciences). To establish HCV replicating cells, Huh-7 cells were transfected with HCV RNA and selected by G418 (Enzo Life Sciences) [164]. Huh-7 cells stably expressing HBx, HCV-2a core, or enhanced green fluorescent protein (EGFP) were generated by way of lentivirus transduction as described previously [164].

13.4.3 Luciferase assay

Cells were lysed in Passive Lysis Buffer (Promega) and the firefly or renilla luciferase activities were measured by Luciferase Assay reagents (Promega) in a TD 20/20 Luminometer (Turner Designs). Firefly or renilla luciferase levels were normalized to the protein concentrations determined by the Bradford assay (Bio-Rad Laboratories). Results were analyzed for statistical differences by the Student's *t* test. A p value of ≤ 0.05 was considered statistically significant.

13.4.4 Western blotting

Proteins were subjected to SDS-PAGE and then blotted onto nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBS and then incubated with a primary antibody overnight at 4 ℃. Membranes were washed and incubated with a secondary antibody for 1 hour at room temperature. After a wash with PBST (PBS+0.1% Tween 20), membranes were scanned using Li-Cor Odyssey scanner (ODY-CLx) and band intensities were determined by Quantity One software (Bio-Rad Laboratories). The following antibodies were used: HBV M (Abcam), HBx (Thermo Scientific), HCV core (Anogen), PTEN (Cell signaling Technology, CST), SREBP-1 (Santa Cruz Biotechnology, SCBT), SREBP-2 (SCBT), β-actin (CST), fibrillarin (Thermo Scientific), Flag (Sigma-Aldrich), and secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (Li-Cor Biosciences).

13.5 Results

13.5.1 HCV does not affect HBV replication in HBV genomic DNA transfected HCV replicating Huh-7 cells

We generated Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$) or HBV payw1.2*7-Luc ($Hygro^R$). Then we confirmed the HBV replication in the cell lines by the detection of the expression of HBV M (Fig. 13.1A). And we only detected the expression of HBx in Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$) but not in Huh-7 cells harboring HBV payw1.2*7-Luc ($Hygro^R$). We observed that the lufierase acitivity in Huh-7 cells harboring HBV payw 1.2-Luc ($Hygro^R$) is significantly higher in comparison to the luciferase activity in Huh-7 cells harboring HBV payw 1.2*7-Luc (HygroR) (Fig. 13.1B). As shown in Fig. 13.1C, we found that in Huh-7 cells harboring HBV payw1.2*7-Luc ($Hygro^R$), without HBx, HBV replication level was significantly lower than in Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$). The data supports our previous data that HBx up-regulates HBV replication [237,284].

Next we wanted to determine whether the HBV genome plasmid DNA replicated in HCV replicating Huh-7 cells. Therefore, payw1.2-Luc (Hygro^R) plasmid or HBV payw1.2*7-Luc (Hygro^R) plasmid were transfected into HCV replicating Huh-7 cells. We used Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) and Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) in which HCV core R50A mutation significantly reduces HCV RNA level (Chapter 9). As shown in Fig. 13.1D, we detected the expression of HBV M protein in HBV payw1.2-Luc (Hygro^R) and HBV payw1.2*7-Luc (Hygro^R) plasmid transfected cells. And we only detected the expression of HBx in HBV payw1.2-Luc (Hygro^R) plasmid transfected cells. Neither wild-type HCV nor mutant HCV altered HBV payw 1.2 or HBV payw 1.2*7 replication; on the other hand, neither HBV payw 1.2 nor HBV payw 1.2*7 affected HCV replication in Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) or in Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (Fig. 13.1E).

13.5.2 The effect of PTEN on HBV and HCV replication in HBV or HCV mono-infections and HBV HCV co-infection

Previously, we reported that the HCV core interacts with PTEN and reduces PTEN level which enhances HCV replication (Chapter 9). On the other hand, several studies have established that HBx also inhibits PTEN expression [289-292]. However, whether or not PTEN regulates HBV replication has not been characterized. Therefore, we were interested in understanding the role of PTEN in HBV and HCV replication in mono-infections and co-infection. Huh-7 cells were co-transfected with plasmids expressing HBV payw1.2-Luc (Hygro^R) (Fig. 13.2A and B) or HBV payw1.2*7-Luc (Hygro^R) (Fig. 13.2C) and plasmids expressing PTEN or empty vector. We observed that overexpression of PTEN significantly inhibited both wild-type HBV and mutant HBV replication. The protein levels of PTEN in wild-type HBV and PTEN co-transfected cells were shown in Fig. 13.2B. Moreover, overexpression of PTEN significantly inhibited HCV replication in Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A), but not in Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (Fig. 13.2D and F), which is consistent with our previous study (Chapter 9). The protein levels of PTEN in PTEN overexpressed wild-type HCV replicating cells were shown in Fig. 13.2E. Then Huh-7 cells harboring wild-type HCV or mutant HCV replicating RNAs were co-transfected with plasmid

expressing PTEN or empty vector and HBV payw1.2-Luc (HygroR) or HBV payw1.2*7-Luc (HygroR) genome plasmids. Overexpression of PTEN inhibited both wild-type HBV and mutant HBV replication in either Huh-7 cells harboring wild-type HCV or mutant HCV replicating RNAs (Fig. 13.2G, I, J and L). While overexpression of PTEN significantly inhibited HCV replication in Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA (Fig. 13.2G and I) but not in Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) RNA (Fig. 13.2J and L). The protein levels of PTEN in wild-type HBV and PTEN transfected Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (Fig. 13.2G) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (Fig. 13.2J) were shown in Fig. 13.2H and K.

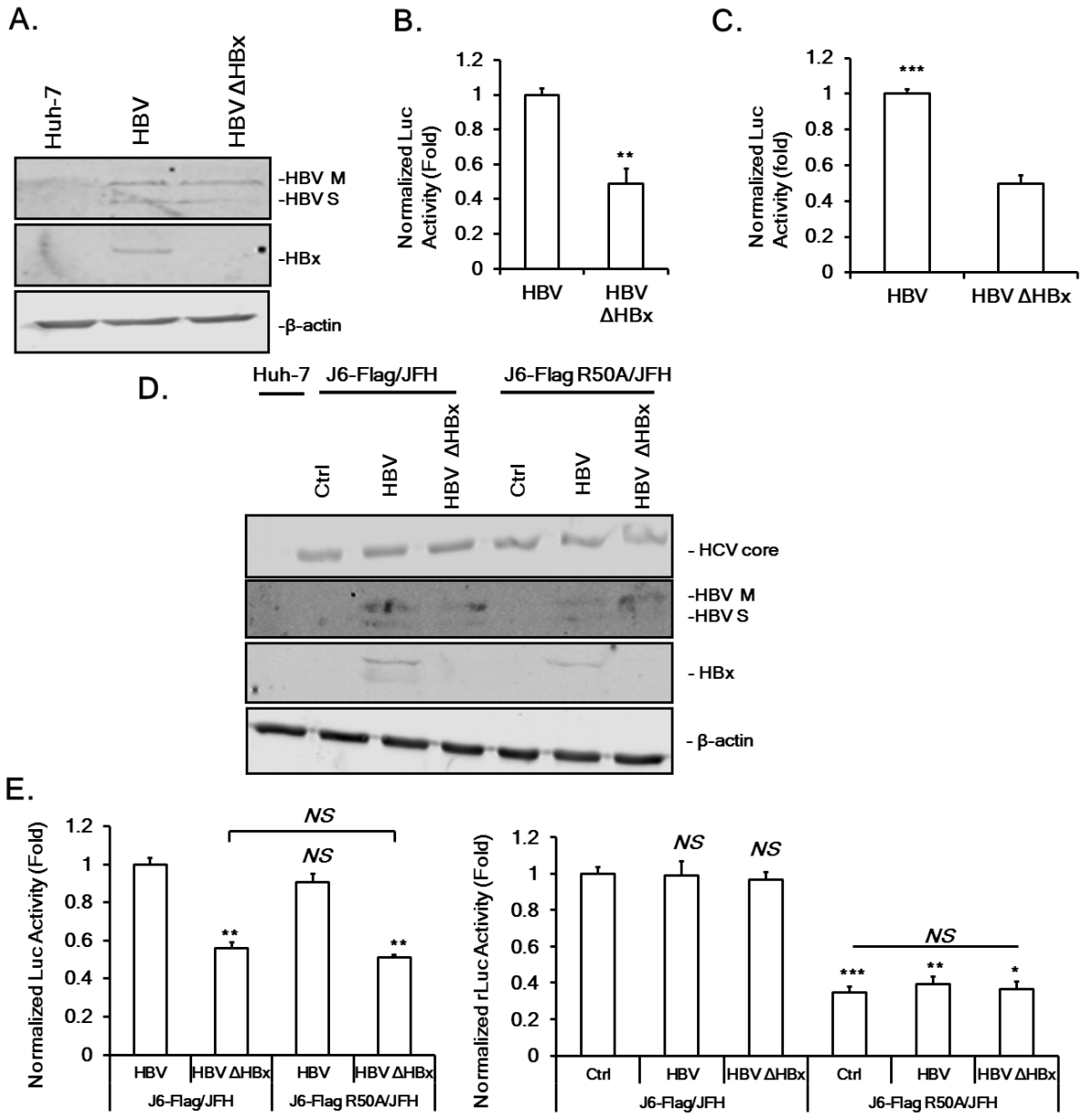
A.
Huh-7
HBV
HBV ΔHBx
-HBV M
-HBV S
-HBx
-β-actin
B.
Normalized Luc Activity (Fold)
HBV
HBV ΔHBx
C.
Normalized Luc Activity (fold)
HBV
HBV ΔHBx
D.
Huh-7
J6-Flag/JFH
J6-Flag R50A/JFH
Ctrl
HBV
HBV ΔHBx
- HCV core
-HBV M
-HBV S
- HBx
- β-actin
E.
Normalized Luc Activity (Fold)
NS
HBV
HBV ΔHBx
J6-Flag/JFH
J6-Flag R50A/JFH
Normalized rLuc Activity (Fold)
Ctrl

Fig. 13.1. HBV and HCV do not interfere with each other in HBV genome plasmid transfected HCV replicating cells. (A, B) The lysates of Huh-7 cells harboring HBV genome payw1.2-Luc (Hygro^R) replicating DNA or HBV genome payw 1.2*7-Luc (Hygro^R) replicating DNA were performed to luciferase activity and Western blotting assay. (A) The expression of HBV M and HBx proteins were determined by Western blotting using anti-HBV M and anti-HBx antibodies. The β-actin levels determined by a β-actin antibody were used as loading controls. This experiment was performed three times (B). Luciferase assay was performed 48 hours after transfection. This experiment was performed three times (C). Huh-7 cells were transfected with HBV genome payw1.2-Luc (Hygro^R) replicating DNA or HBV genome payw 1.2*7-Luc (Hygro^R) replicating DNA. Luciferase assay was performed 48 hours after transfection. This experiment was performed three times (D, E). Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1(p7-rLuc-2A) replicating RNA or HCV-2a J6 core-Flag R50A/JFH-1(p7-rLuc-2A) replicating RNA were transfected with plasmids containing HBV genome payw1.2-Luc (Hygro^R), HBV genome payw1.2*7-Luc (Hygro^R) or vector (D). The expression of HCV core, HBV M and HBx proteins were determined by Western blotting using anti-Flag, anti-HBV M and anti-HBx antibodies. The β-actin levels determined by a β-actin antibody were used as loading controls. This experiment was performed two times (E). Dual luciferase assay was performed 48 hours after transfection. This experiment was performed three times (B, C, E). Luciferase activity and dual luciferase assay were measured and normalized to total protein amount. Statistical differences between samples were analyzed by Student's *t* test and demonstrated as ** if $p \leq 0.01$, *** $p \leq 0.001$ or *NS* for not significant.

To further determine the effect of PTEN on HBV and HCV replication, we used PTEN shRNA to knock down PTEN. We found that PTEN knockdown significantly enhanced both wild-type (Fig. 13.3A, G and J) and mutant HBV (Fig. 13.3B, I and L) replication in HBV and PTEN shRNA co-transfected cells. On the other hand, PTEN knockdown significantly enhanced HCV replication in Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA (Fig. 13.3D, G and I) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) RNA (Fig. 13.3F, J and L). Interestingly, we observed that PTEN knockdown enhanced HBV replication around 4-fold (Fig. 13.3A and C) in HBV and PTEN shRNA transfected Huh-7 cells while only enhance HBV replication around 1.5-fold in HBV and PTEN shRNA transfected HCV replicating Huh-7 cells (Fig. 13.3G, I, J and L). We found that the protein level of PTEN reduced around 70% in HBV and PTEN shRNA transfected Huh-7 cells in comparison to HBV and control shRNA transfected Huh-7 cells (Fig. 13.3B); whereas, the protein levels of PTEN reduced around 40% to 50% in PTEN knockdown HCV replicating Huh-7 cells (Fig. 13.3E, H and K). The higher efficiency of PTEN shRNA in Huh-7 cells may result in the stronger effect on HBV replication.

Overall, these data indicate that PTEN inhibits HBV and HCV replication in HBV or HCV mono-infections and HBV HCV co-infection.

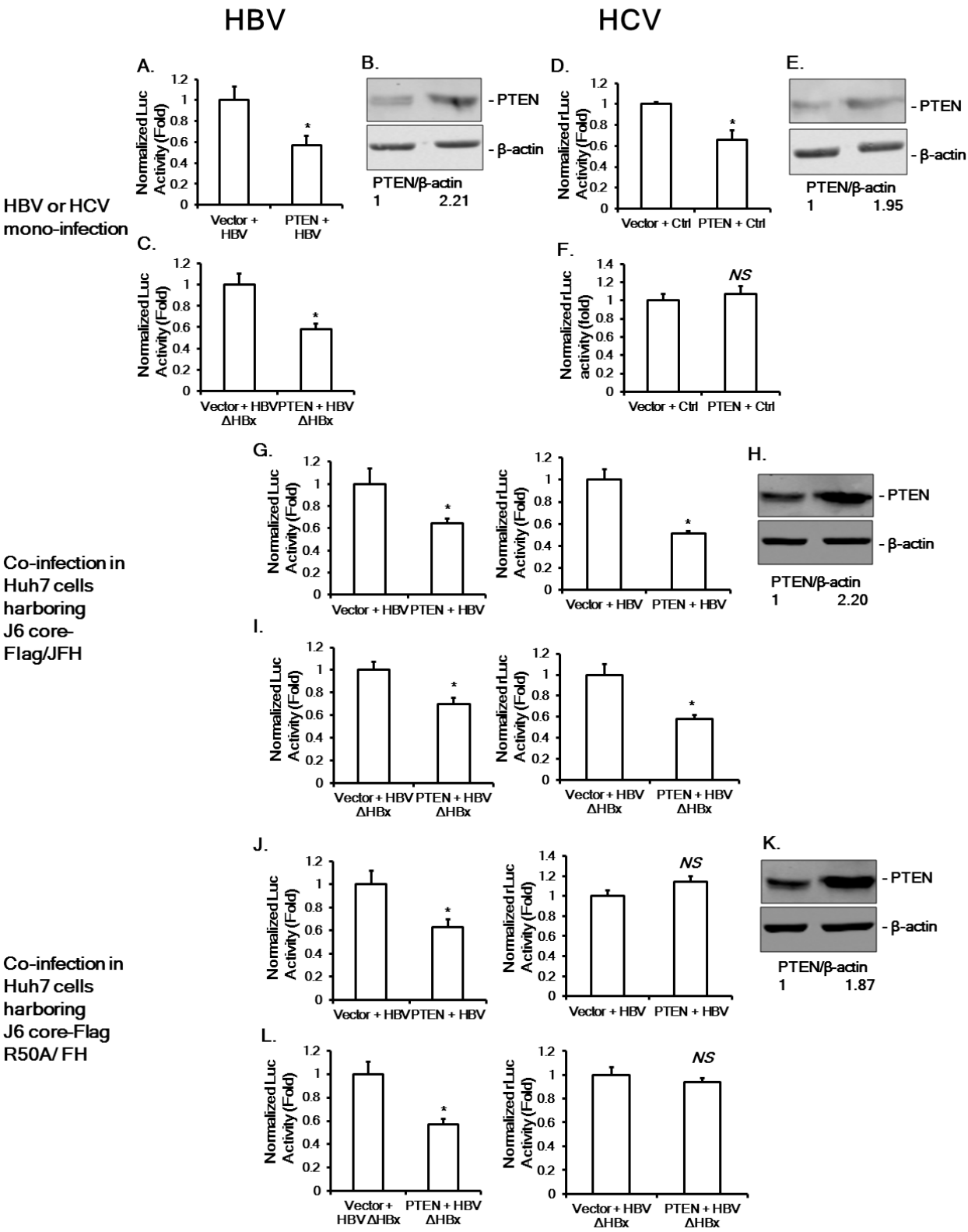
HBV
HCV
HBV or HCV mono-infection
Co-infection in Huh7 cells harboring J6 core-Flag/JFH
Co-infection in Huh7 cells harboring J6 core-Flag R50A/ FH
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.
Normalized Luc Activity (Fold)
Normalized rLuc Activity (Fold)
Normalized rLuc activity (fold)
Vector + HBV
PTEN + HBV
Vector + HBV ΔHBx
PTEN + HBV ΔHBx
Vector + Ctrl
PTEN + Ctrl
- PTEN
- β-actin
PTEN/β-actin
1
2.21
1.95
2.20
1.87
NS

Fig. 13.2. Overexpression of PTEN inhibits HBV and HCV replication in HBV or HCV mono-infections and HBV HCV co-infection. Huh-7 cells (A - C), Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (D, E, G, H and I) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (F, J, K and L) were co-transfected with plasmids expressing Flag-PTEN or empty vector and plasmids containing HBV genome payw1.2-Luc ($Hygro^R$), HBV genome payw1.2*7-Luc ($Hygro^R$) or vector. (A, C, D, F, G, I, G, L) Luciferase activity was measured 48 hours after transfection and normalized to total protein amount. Luciferase activities were expressed as fold changes relative to vector control. Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ or *NS* for not significant. These experiments were performed three times. The protein levels of PTEN in A, D, G and J were shown in B, E, H and K. The total protein levels of PTEN were analyzed by Western blotting using anti-PTEN antibody. The β-actin determined by anti-β-actin antibody was used as loading controls. The intensities of the bands were analyzed by the Quantity One program. These experiments were performed two times.

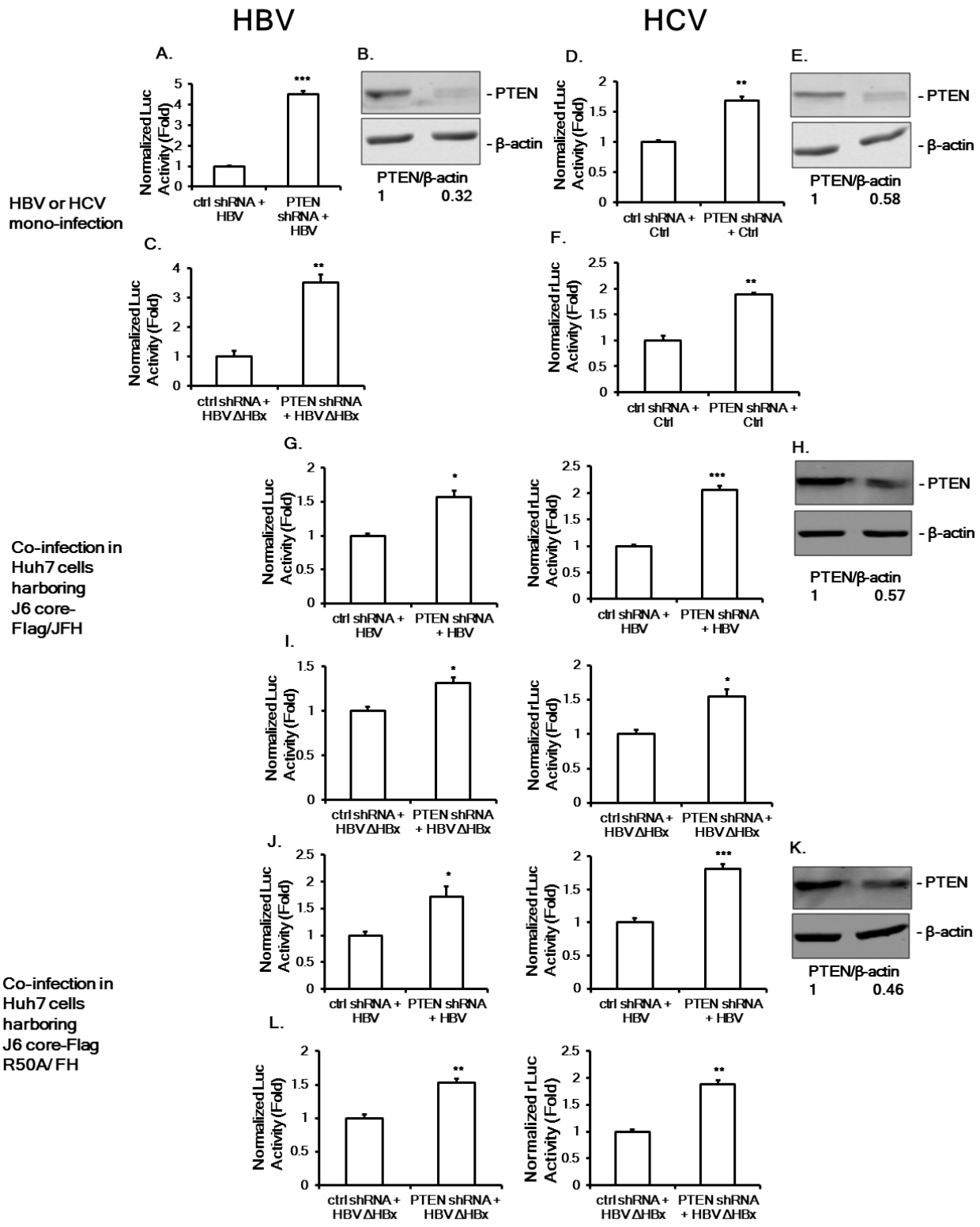
HBV
HCV
A.
B.
D.
E.
HBV or HCV mono-infection
C.
F.
G.
H.
Co-infection in Huh7 cells harboring J6 core-Flag/JFH
I.
J.
K.
Co-infection in Huh7 cells harboring J6 core-Flag R50A/ FH
L.
Normalized Luc Activity (Fold)
Normalized rLuc Activity (Fold)
ctrl shRNA + HBV
PTEN shRNA + HBV
ctrl shRNA + HBVΔHBx
PTEN shRNA + HBVΔHBx
ctrl shRNA + Ctrl
PTEN shRNA + Ctrl
- PTEN
- β-actin
PTEN/β-actin
1
0.32
0.58
0.57
0.46

Fig. 13.3. PTEN knockdown enhances both HBV and HCV replication in HBV or HCV mono-infections and HBV HCV co-infection. Huh-7 cells (A - C), Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (D, E, G, H and I) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (F, J, K and L) were co-transfected with plasmids expressing non-silencing control shRNA or shRNA targeting the 3'-UTR of PTEN and plasmids containing HBV genome payw1.2-Luc ($Hygro^R$), HBV genome payw1.2*7-Luc ($Hygro^R$) or vector. (A, C, D, F, G, I, G, L) Luciferase activity was measured 48 hours after transfection and normalized to total protein amount. Luciferase activities were expressed as fold changes relative to vector control. Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ or *NS* for not significant. These experiments were performed three times. The protein levels of PTEN in A, D, G and J were shown in B, E, H and K. The total protein levels of PTEN were analyzed by Western blotting using anti-PTEN antibody. The β-actin determined by the anti-β-actin antibody was used as loading controls. The intensities of the bands were analyzed by the Quantity One program. These experiments were performed two times.

13.5.3 The effect of PTEN and SREBP-1 on HBV and HCV replication in HBV or HCV mono-infections and HBV HCV co-infection

PTEN inhibits PI3K/Akt pathway through its lipid phosphatase activity [45,49] and thus inhibits SREBP-1 activation which is a downstream factor of Akt [210]. We next wanted to determine whether or not PTEN regulates HBV and HCV replication through modulating SREBP-1 activation. We used PTEN shRNA and SREBP-1 microRNA (miRNA) (which was used in Chapters 3 and 7) to knockdown PTEN and/or SREBP-1 in HBV or HCV replicating Huh-7 cells. As shown in Fig. 13.4A and B, knocking down PTEN significantly enhanced HBV replication in Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$), while knocking down SREBP-1 significantly inhibited HBV replication. In the PTEN shRNA and SREBP-1 miRNA co-transfected cells, the protein levels of PTEN and nuclear SREBP-1 were down-regulated around 50% in comparison to the protein levels in non-silencing shRNA and non-silencing miRNA co-transfected cells (Fig. 13.4B), which led to inhibiting HBV replication (Fig. 13.4A). This result indicates that PTEN modulates HBV replication through regulating SREBP-1 activation. However, in Huh-7 cells harboring HBV payw1.2*7 ($Hygro^R$), only knocking down PTEN significantly enhanced HBV replication; knocking down SREBP-1 did not alter HBV replicating (Fig. 13.4C). Overall, these results indicate that HBx plays a role in PTEN/SREBP-1 modulated HBV replication. As shown in Fig. 13.4D to F, in Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA and Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) RNA, PTEN knockdown significantly enhanced HCV replication; SREBP-1 knockdown significantly inhibited HCV replication; however, knocking down SREBP-1 does not affect the PTEN-modulated HCV replication. The data show that PTEN and SREBP-1 can modulate HCV replication. However, PTEN does not modulate HCV replication through the inhibition of SREBP-1 activation.

Next, we wanted to determine the role of PTEN and SREBP-1 in HBV and HCV replication in HBV HCV co-replicating cells. HCV replicating Huh-7 cells were first transfected with HBV genome plasmid DNA and then co-transfected with PTEN shRNA and SREBP-1 miRNA. The protein levels of PTEN were down-regulated in PTEN shRNA transfected cells in comparison to non-silencing shRNA transfected cells; the protein levels of nuclear SREBP-1 were modulated by PTEN shRNA and SREBP-1 miRNA as expected (Fig. 13.5C). As shown in

Fig. 13.5A and B, knocking down PTEN enhanced HBV replication; however, knocking down SREBP-1 inhibited wild-type HBV replication. On the other hand, only SREBP-1 knockdown inhibited HCV replication and PTEN knockdown enhanced HCV replication irrespective of SREBP-1 knockdown. These data suggest that PTEN and SREBP-1 have similar effect on HBV and HCV replication in HBV HCV co-infection as well as in HBV or HCV mono-infections.

13.5.4 The effect of HBx and HCV core on PTEN and SREBP-1

In order to determine the effect of HBx and HCV core on PTEN and SREBP-1, we established Huh-7 cells stably expressing HBx, HCV core or EGFP. We observed that the protein levels of PTEN were significantly lower and the protein levels of nuclear SREBP-1 were significantly higher in Huh-7 cells stably expressing HBx and Huh-7 cells stably expressing HCV core in comparison to the PTEN protein level and SREBP-1 protein level in EGFP-expressing Huh-7 cells (Fig. 13.6A). The data indicate that both the HBx and HCV core can down-regulate PTEN and up-regulate the SREBP-1 at the protein level. Then we wanted to determine whether the HBx and HCV core synergistically regulate PTEN and SREBP-1. Plasmid expressing HBx was transfected into HCV core-expressing Huh-7 cells. We found that the protein levels of PTEN and SREBP-1 was not further modulated after HBx expression in Huh-7 cells stably expressing HCV core in comparison to the protein levels of PTEN and SREBP-1 in empty vector or EGFP transfected cells (Fig. 13.6B). The data imply that the effects of HBx and HCV core on PTEN and SREBP-1 are not additive.

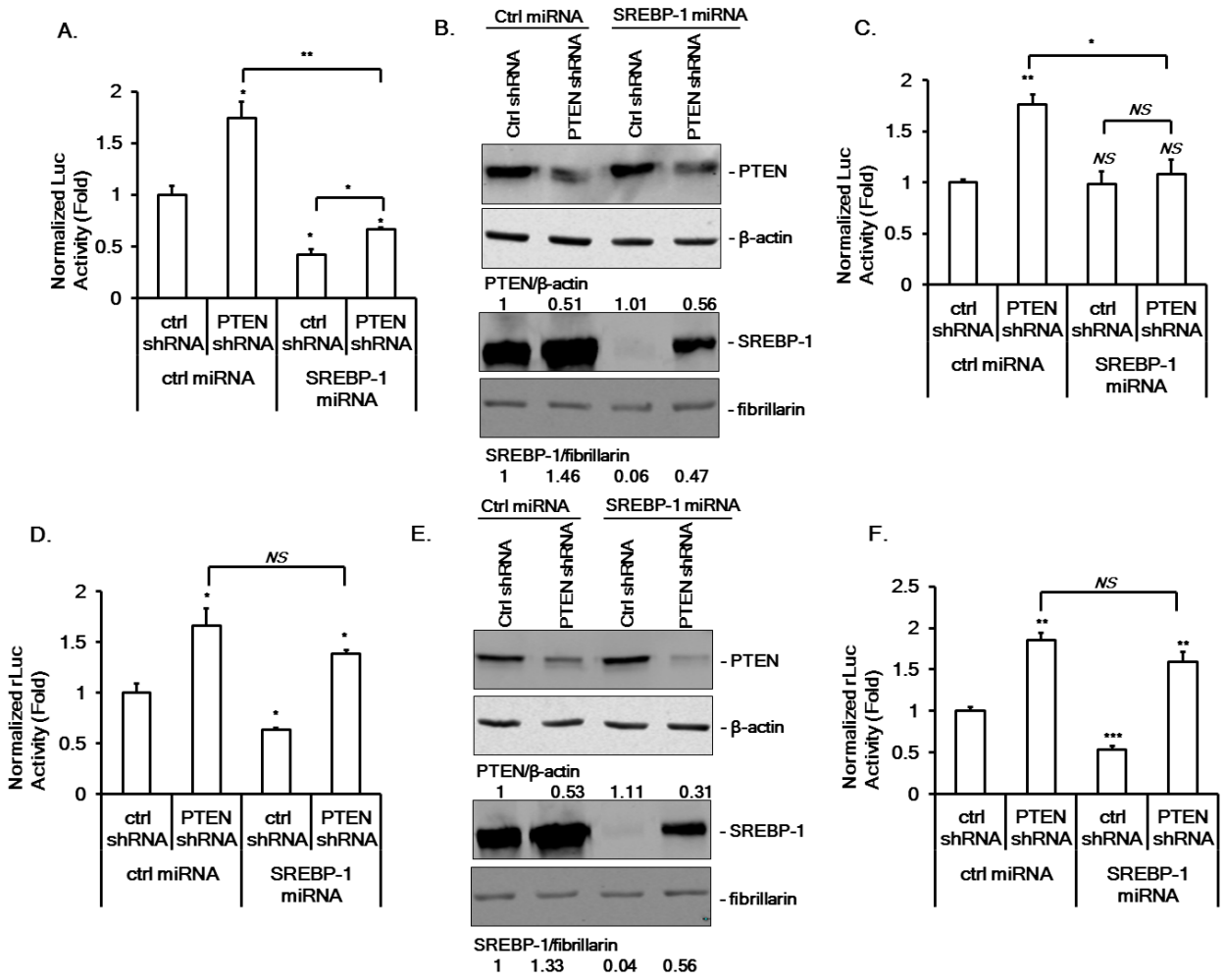

Fig. 13.4. The role of PTEN/SREBP-1 pathway on HBV and HCV replication in HBV or HCV mono-infected cells. Huh-7 cells HBV genome payw1.2-Luc (Hygro^R) (A, B), Huh-7 cells harboring HBV genome payw1.2*7-Luc (Hygro^R) (C), Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (D, E) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (F) were co-transfected with plasmids expressing non-silencing control shRNA or shRNA targeting the 3'-UTR of PTEN and non-silencing control miRNA or SREBP-1-targeting miRNA. At 48 hours after transfection, (A, C, D, F) Luciferase activities were expressed as fold changes relative to vector control. Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ or *NS* for not significant. These experiments were performed three times. (B, E) The total protein levels of PTEN were analyzed by Western blotting using anti-PTEN antibody. The β-actin determined by anti-β-actin antibody was used as loading controls. The nuclear protein levels of SREBP-1 were analyzed by Western blotting using anti-SREBP-1 antibody. The fibrillarin levels determined by an anti-fibrillarin antibody were used as loading controls. The intensities of the bands were analyzed by the Quantity One program. These experiments were performed two times.

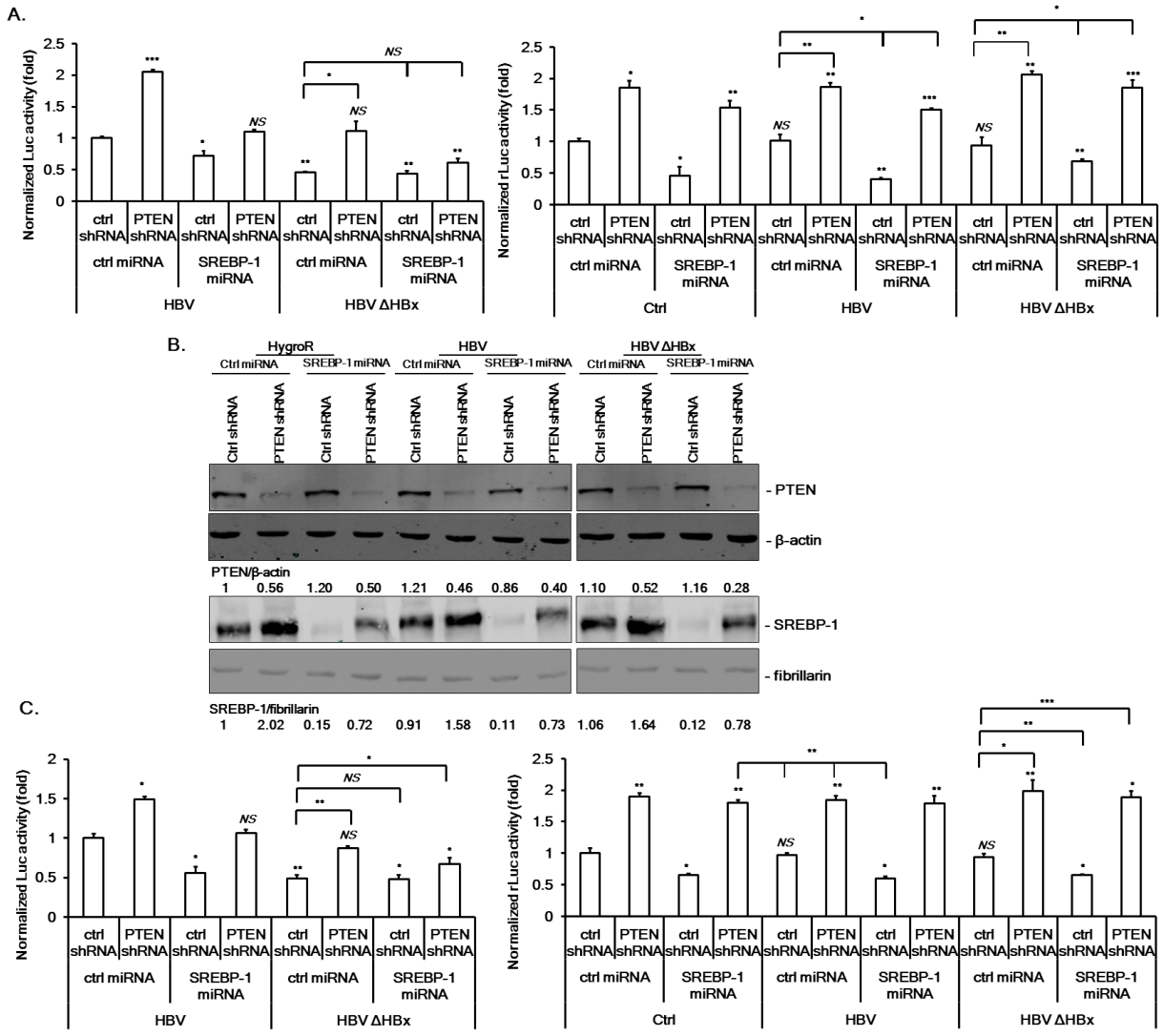
A.
Normalized Luc activity (fold)
Normalized rLuc activity (fold)
ctrl shRNA
PTEN shRNA
ctrl miRNA
SREBP-1 miRNA
HBV
HBV ΔHBx
Ctrl
NS
B.
HygroR
HBV
HBV ΔHBx
Ctrl miRNA
SREBP-1 miRNA
Ctrl shRNA
PTEN shRNA
- PTEN
- β-actin
PTEN/β-actin
1 0.56 1.20 0.50 1.21 0.46 0.86 0.40 1.10 0.52 1.16 0.28
- SREBP-1
- fibrillarin
SREBP-1/fibrillarin
1 2.02 0.15 0.72 0.91 1.58 0.11 0.73 1.06 1.64 0.12 0.78
C.
Normalized Luc activity (fold)
Normalized rLuc activity (fold)
HBV
HBV ΔHBx
Ctrl

Fig. 13.5. The role of PTEN/SREBP-1 pathway on HBV and HCV replication in HBV HCV co-infected cells. Huh-7 cells harboring HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) (A, B) or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) (C) were transfected with plasmids containing HBV genome payw1.2-Luc (HygroR), HBV genome payw1.2*7-Luc (HygroR) or HygroR gene. At 24 hours after transfection, cells were then co-transfected with plasmids expressing non-silencing control shRNA or shRNA targeting the 3'-UTR of PTEN and non-silencing control miRNA or SREBP-1-targeting miRNA. At another 48 hours after transfection, (A, B) Luciferase activities were expressed as fold changes relative to vector control. Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ or *NS* for not significant. These experiments were performed three times. (C) The total protein levels of PTEN were analyzed by Western blotting using anti-PTEN antibody. The β-actin determined by anti-β-actin antibody was used as loading controls. The nuclear protein levels of SREBP-1 were analyzed by Western blotting using anti-SREBP-1 antibody. The fibrillarin levels determined by an anti-fibrillarin antibody were used as loading controls. The intensities of the bands were analyzed by the Quantity One program. These experiments were performed two times.

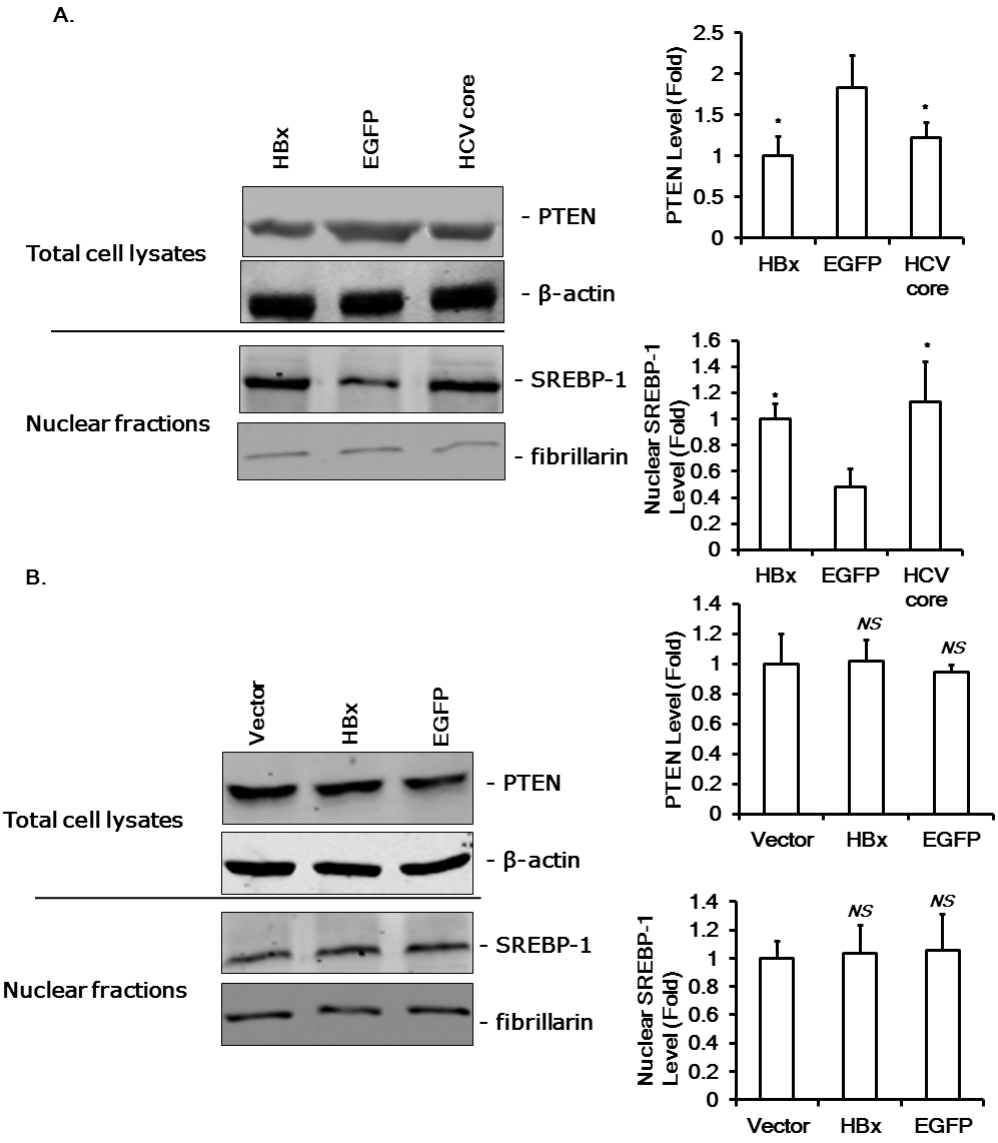

Fig. 13.6. HBx and HCV core reduce PTEN protein level and enhance nuclear SREBP-1 protein level. (A) The total protein levels of PTEN and nuclear protein levels of SREBP-1 in Huh-7 cells stably expressing HBx, EGFP or HCV core were analyzed by Western blotting using anti-PTEN and anti-SREBP-1 antibodies. The β-actin and fibrillarin levels determined by anti-β-actin and anti-fibrillarin antibodies were used as loading controls. (B) Huh-7 cells stably expressing HCV core were transfected with plasmids expressing HBx-3xmyc, EGFP-3xmyc or empty vector. The total protein levels of PTEN and nuclear protein levels of SREBP-1 in total cell lysates or in nuclear fractions were analyzed by Western blotting using anti-PTEN and anti-SREBP-1 antibodies. The β-actin and fibrillarin levels determined by anti- β-actin and anti-fibrillarin antibodies were used as loading controls. (A, B) The intensities of the bands were analyzed by the Quantity One program. The statistical differences between samples were demonstrated as * if $p \leq 0.05$ or *NS* for not significant. Error bars indicated standard error of the mean (n=3).

13.5.5 HBV enhanced HCV translation and replication only in the early stage of HCV infection

Previously, we have shown that HBV and HCV can replicate without interfering with each other in HCV replicating Huh-7 cells transfected with HBV genomic DNA. Next, we wanted to study HCV replicates in HBV replicating cells. Huh-7 cells, Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) or HBV payw 1.2*7 ($Hygro^R$) were transfected with wild-type or R50A mutant HCV replicating RNA. As shown in Fig. 13.7A, HCV did not affect HBV replication in Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) or HBV payw 1.2*7 ($Hygro^R$) from 4 - 72 hours after transfection. However, at 4 hours after transfection, the value of rLuc activities of mutant HCV were significantly higher in Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) or HBV payw 1.2*7 ($Hygro^R$) than in Huh-7 cells (Fig. 13.7A). Therefore, we hypothesized that HBV may enhance HCV translation. To examine whether HBV affects HCV translation, Huh-7 cells were co-transfected with replication-deficient wild-type or R50A mutant HCV genomic RNA and plasmids expressing HBV proteins or empty vectors. We found that HBV S slightly but significantly enhanced both wild-type and mutant HCV translation (Fig. 13.7B and C). The expression of HBV proteins was shown in Fig. 13.7D. We also noticed that, in Fig. 13.7A, at 24 hours and 48 hours after transfection, HCV replication level was higher in HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA transfected Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) in comparison to HCV replication levels in HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA transfected Huh-7 cells or Huh-7 cells harboring HBV payw 1.2*7 ($Hygro^R$). It may indicate that HBV enhances the HCV replication. To futher determine the effect of HBV on HCV replication, Huh-7 cells, Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) or HBV payw 1.2*7 ($Hygro^R$) were infected with HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) virus. We found that only at 48 hours post infection, HCV replication level was higher in HCV infected Huh-7 cells harboring HBV payw 1.2 ($Hygro^R$) in comparison to HCV replication levels in HCV infected Huh-7 cells or Huh-7 cells harboring HBV payw 1.2*7 ($Hygro^R$) (Fig. 13.7E). Overall, the data indicate that HBV enhances HCV replication only in the early period of HCV infection. HBx plays an important role in the regulation of HCV replication.

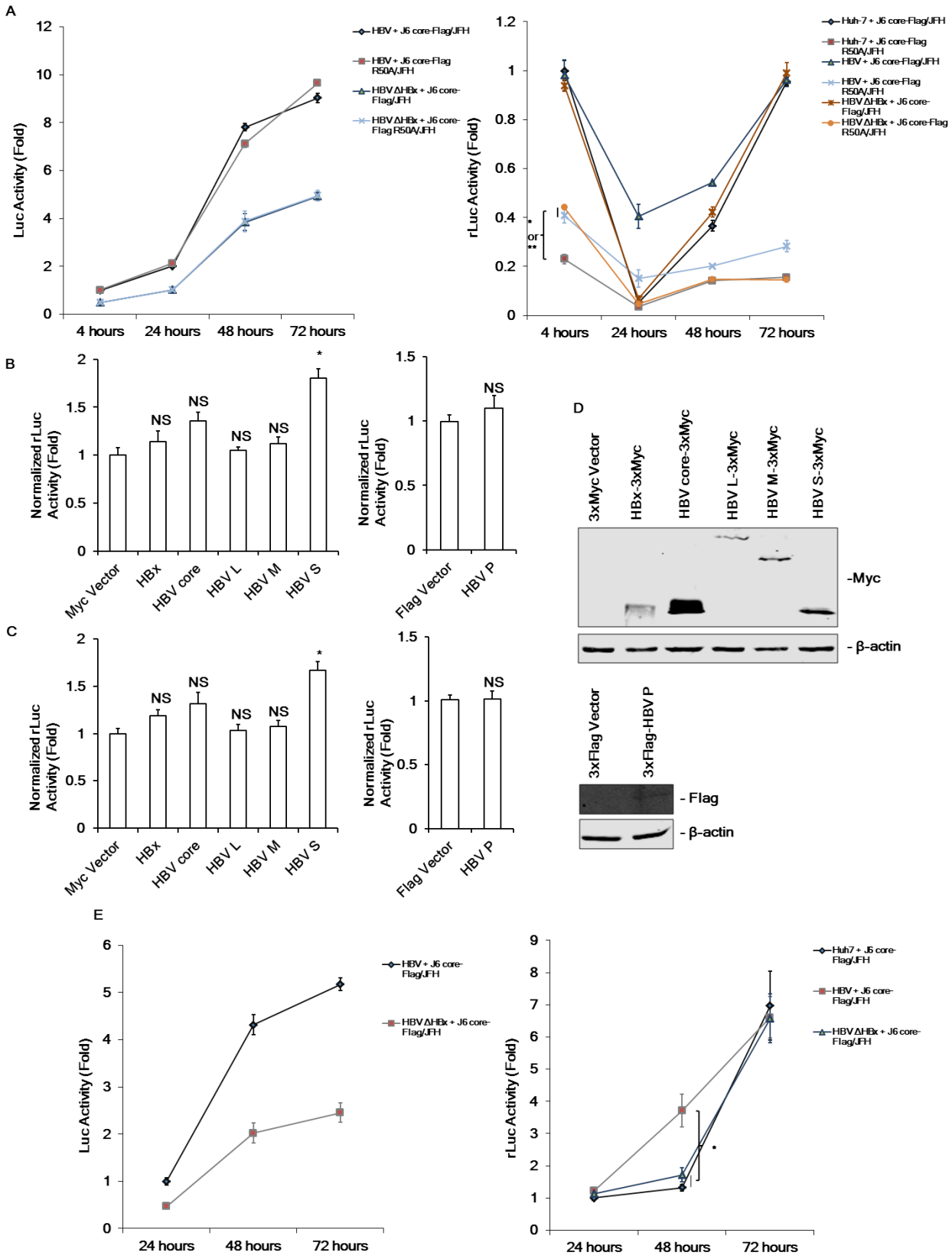
A
Luc Activity (Fold)
HBV + J6 core-Flag/JFH
HBV + J6 core-Flag R50A/JFH
HBV ΔHBx + J6 core-Flag/JFH
HBV ΔHBx + J6 core-Flag R50A/JFH
4 hours
24 hours
48 hours
72 hours
rLuc Activity (Fold)
Huh-7 + J6 core-Flag/JFH
Huh-7 + J6 core-Flag R50A/JFH
HBV + J6 core-Flag/JFH
HBV + J6 core-Flag R50A/JFH
HBV ΔHBx + J6 core-Flag/JFH
HBV ΔHBx + J6 core-Flag R50A/JFH
B
Normalized rLuc Activity (Fold)
NS
Myc Vector
HBx
HBV core
HBV L
HBV M
HBV S
Flag Vector
HBV P
C
D
3xMyc Vector
HBx-3xMyc
HBV core-3xMyc
HBV L-3xMyc
HBV M-3xMyc
HBV S-3xMyc
-Myc
- β-actin
3xFlag Vector
3xFlag-HBV P
- Flag
- β-actin
E

Fig. 13.7. HBV enhances HCV translation in HCV RNA transfected HBV replicating cells and up-regulates early stage of HCV replication in HCV infected HBV replicating cells. Huh-7 cells, Huh-7 cells harboring HBV genome payw1.2-Luc (Hygro^R) or Huh-7 cells harboring HBV genome payw1.2*7-Luc (Hygro^R) were transfected with HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) RNA or Huh-7 cells harboring HCV-2a J6 core-Flag R50A/JFH-1 (p7-rLuc-2A) RNA. At 4, 24, 48 and 72 hours after transfection, luciferase activities were expressed as fold changes relative to vector control. Huh-7 cells were co-transfected with HBV proteins or empty vector and a monocistronic HCV-2a translation rLuc reporter RNA (B) or a full-length HCV-2a J6/JFH-1 (p7-rLuc-2A) GNN RNA (C). At 48 hours after transfection, luciferase activities were expressed as fold changes relative to vector control. (D) Huh-7 cells were transfected with HBV proteins or empty vector. At 48 hours after transfection, the expression of the proteins was determined by Western blotting assay using anti-Myc and anti-Flag antibodies. (E) Huh-7 cells, Huh-7 cells harboring HBV genome payw1.2-Luc (Hygro^R) or Huh-7 cells harboring HBV genome payw1.2*7-Luc (Hygro^R) were infected with HCV-2a J6 core-Flag/JFH-1 (p7-rLuc-2A) virus at a multiplicity of infection (MOI) of 1. At 24, 48, and 72 hours after transfection, luciferase activities were expressed as fold changes relative to vector control. (A, B, C and E) Statistical differences between samples were analyzed by Student's *t* test and demonstrated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ or *NS* for not significant.

13.6 Discussion

HBV HCV co-infection includes four clinical features: acute HBV/HCV co-infection, HCV superinfection in chronic hepatitis B (CHB) patients, HBV superinfection in chronic hepatitis C (CHC) patients, and occult hepatitis B (OHB) [93,293,294]. Therefore, how HBV and HCV replicate in the cells in the early period after infection is worthy of study. In this study, we constructed plasmids HBV payw1.2-Luc ($Hygro^R$) or HBV payw1.2*7-Luc ($Hygro^R$) and then established Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$) and Huh-7 cells harboring HBV payw1.2*7-Luc ($Hygro^R$). In HBV genome plasmid transfected HCV replicating Huh-7 cells, we observed that HBV and HCV can replicate in HBV genomic DNA transfected Huh-7 cells without interfering with each other (Fig. 13.1). On the other hand, in HCV genomic replicon RNA transfected HBV replicating Huh-7 cells, we found that HBV enhanced HCV translation and replication just within 48 hours after HCV infection (Fig. 13.7). We observed that HBV S protein is essential for up-regulating HCV translation and HBx is required for enhancing HCV replication.

HBV or HCV mono-infections and HBV HCV co-infection are risk factors of developing HCC [283]. To study the potential mechanism of how HBV and HCV infections cause HCC, we focused on a tumor suppressor, PTEN. Interestingly, we found that PTEN can inhibit HBV or HCV replication through different mechanisms. When we used PTEN shRNA and SREBP-1 miRNA to modulate the protein level of PTEN and nuclear SREBP-1, we found that HBV replication was inversely correlated with the SREBP-1 level in the nucleus (Fig. 13.4A and B). PTEN regulates SREBP-1 activation through its lipid phosphatase activity. Moreover, HBx is required in this process (Fig. 13.4C), which supports our previous study [284]. In HCV replicating Huh-7 cells, although knocking down SREBP-1 resulted in inhibiting HCV replication, PTEN knockdown counteracted this inhibition and even enhanced HCV replication (Fig. 13.4C and D). It implies that SREBP-1 activation is important for HCV replication. However, PTEN does not regulate HCV replication through modulating SREBP-1. Moreover, we found that PTEN and SREBP-1 play similar roles in regulating HBV and HCV replicating in HBV or HCV mono-infections as in HBV HCV co-infection (Fig. 13.4 and 13.5).

Since HBx plays an important role in PTEN/SREBP-1 mediated HBV replication (Fig. 13.4A to C), and our previous study has shown that the interaction between HCV core and PTEN

is involved in HCV replication. We proposed that HBx and HCV core may be essential for regulating PTEN and SREBP-1 which leads to modulating HBV and HCV replication in HBV HCV co-infection. First, we found that both HBx and HCV core can inhibit PTEN and stimulate SREBP-1 activation (Fig. 13.6A). Next, we observed that overexpression of HBx in Huh-7 cells stably expressing HCV core did not alert the protein level of PTEN and nuclear SREBP-1 (Fig. 13.6B). This result indicates that HBx and HCV core may play an independent role in regulating PTEN and SREBP-1 in HBV HCV co-infection. It is the potential mechanism in explaining why PTEN and SREBP-1 play the same role in HBV or HCV mono-infections as in HBV HCV co-infection. It also is the potential mechanism in explaining why HBV enhances HCV replication only in the early period of HCV infection in HCV infected HBV replicating Huh-7 cells (Fig. 13.7E). We have previously shown that HCV core can interact with PTEN and reduce the protein level of PTEN which enhanced HCV replication (Chapter 9). In Huh-7 cells harboring HBV payw1.2-Luc ($Hygro^R$), the protein level of PTEN may be lower than in Huh-7 cells or in Huh-7 HBV payw1.2*7-Luc ($Hygro^R$) since HBx inhibits the protein level of PTEN. Therefore, in the early period of HCV infection, the HBx mediated low protein level of PTEN may enhance HCV replication. After that, HCV core may play the same role as HBx, thus HBV no longer enhances HCV replication.

In summary, we studied an HCC-related signaling pathway, the PTEN/SREBP-1 pathway, in HBV or HCV mono-infections and HBV HCV co-infection. We demonstrated that both HBx and HCV core inhibit the protein level of PTEN and enhance SREBP-1 activation, and modulating the protein level of PTEN and nuclear SREBP-1 can regulate HBV and HCV replications. We also studied the early the role of HBV and HCV replication in HBV genomic DNA transfected HCV replicating cells or HCV genomic RNA transfected HBV replicating cells. This study contributes to understand the pathnogenesis in HCV superinfection in CHB or HBV superinfection in CHC. In a further study, we plan to establish HBV HCV co-replicating Huh-7 cells and study the effect of PTEN/SREBP-1 pathway on HBV and HCV replication in HBV HCV co-replicating cells. The study may contribute to develop of the new therapy of HBV HCV chornic co-infection.

13.7 Acknowledgements

This work was supported by grants from Canadian Institutes of Health Research, Saskatchewan Health Research Foundation, and Natural Sciences and Engineering Research Council of Canada to QL. QW is a recipient of a Ph.D. The research leading to these results has also received funding from CIHR (FRN# NHC-142832) and the Public Health Agency of Canada (PHAC) in the form of a Ph.D. scholarship to QW.

14.0 GENERAL DISCUSSION, CONCLUSION AND FUTURE EXPERIMENTS

14.1 General Discussion

HBV infection causes acute and chronic liver disease and has been proved to be a high risk factor for the development of HCC [295]. Previous studies have shown that HBx functions as a transcriptional transactivator of host genes and promotes cell growth and HCC [18,100]. SREBPs are transcription factors which belong to the bHLH-ZIP family [67]. A previous study has demonstrated that in HBV-associated HCC specimens, the expression of SREBP-1 is significantly increased [110]. Therefore, we hypothesized that if HBx regulates SREBP-1 activity, it may occur at different levels including SREBP gene transcription, SREBP cleavage and nuclear localization. In Chapter 3, we first determined that the expression of HBx enhances the total SREBP-1 protein level and the nuclear SREBP-1a protein level (Fig. 3.1). Then we demonstrated that HBx modulates SREBP-1 at the transcription level (Fig. 3.2). Interestingly, we observed that SREBP-1a promoter activity is increased around 3-fold in HBV genomic DNA transfected cells in comparison to empty vector transfected cells; however, in HBV ΔHBx genomic DNA transfected cells, SREBP-1a promoter activity is also slightly increased (Fig. 3.2E). This result may indicate that besides HBx, other HBV protein(s) may also enhance SREBP-1a promoter activity. The mechanism is not understood at this time. The HBV genome plasmid payw1.2 can express the HBV pregenomic RNA and HBV proteins. The plasmid with a mutant which containing a stop codon affect HBx amino acid position 7 can express other HBV proteins but not HBx [200]. Therefore, these HBV genome plasmids are widely used models for studying HBV replication. HBV gene expression is regulated by two HBV enhancers [218]. HBx also activates HBV enhancers and thus increases HBV replication [11,12]. We found that after knocking down SREBP-1, HBx is no longer able to transactivate the HBV enhancer II/core promoter (Fig. 3.4). It indicates that SREBP-1 plays an important role in the HBx-associated activation of the HBV enhancer II/core promoter. Therefore, we were interested in determining the mechanisms of how HBx regulates SREBP-1 and HBV enhancers. Since transcription of shRNA targets one specific gene and results in gene knockdown [296]. We planned to use

SREBP-1 shRNA to knockdown SREBP-1. However, the SREBP-1 shRNA we designed did not reduce the SREBP-1 protein level. Previous study has reported that shRNA sometimes did not lead to efficient gene knockdown [297]. Then we tested whether the SREBP-1 miRNA we designed can reduce SREBP-1 protein level. As shown in Fig. 3.4A, the SREBP-1 miRNA reduced both the total and nuclear SREBP-1 protein levels.

HBx contains 154 amino acids with a molecular weight of 17 kDa and is localized in the nucleus, cytoplasm, and mitochondria [123-125]. Different subcellular localizations of HBx display different transactivational activities [237]. HBx has been reported to induce cell proliferation through modulation of the PTEN/PI3K/Akt pathways [290,298]. Moreover, SREBP-1 is the downstream factor of the PI3K/Akt [176], and activation of SREBP-1a by HBx leads to lipid cell proliferation in human liver cells [110,138]. Previous studies have demonstrated that the transactivation of SREBP-1a and FASN by HBx induces lipid accumulation [109,215]. In Chapter 5, we showed that nuclear HBx, rather than wild-type HBx or cytoplasmic HBx, has stronger effects on activating SREBP-1a and FASN transcription, increasing intracellular lipid accumulation and cell proliferation (Fig. 5.2). These results indicate that HBx has to localize in the nucleus to transactivate SREBP-1a and FASN and induce lipid accumulation and cell proliferation. Moreover, we also observed that wild-type HBx and nuclear HBx but not cytoplasmic HBx enhances HBV enhancer I/X promoter activity and HBV mRNA level in the context of HBV replication; however, only wild-type HBx enhances HBV enhancer II/core promoter activity (Fig. 5.3). These results imply that nuclear HBx stimulates HBV replication through up-regulation of HBV enhancer I/X promoter activity. However, the mechanism of how HBx regulates HBV enhancer II/core promoter activity is still not clear.

Truncated forms of HBx are more commonly found in HBV-associated tumor tissues [130-132]. The pathobiological significance of this finding is not well understood. Previous studies have demonstrated that the C-terminal region of HBx plays important roles in HBx stability [132-134]. In addition, the C-terminal region of HBx is involved in HBx-modulated HBV replication in cell culture [12,133,135]. Therefore, we were interested in whether different truncated forms of HBx regulate SREBP-1 and HBV enhancers differentially. Previous studies have reported that HBx plays different role in regulation of HBV replication in HepG2 or Huh-7 cells [196,219]. Therefore, we used both HepG2 and Huh-7 cells to study how HBx regulates HBV replication.We observed that HBx aa. 1-127 can only stimulate SREBP-1c transcription but

not SREBP-1a transcription; on the other hand, HBx aa. 43-154 can only enhance SREBP-1a transcription but not SREBP-1a transcription (Fig. 7.1). Moreover, HBx aa. 1-127 can only up-regulate HBV enhancer I/X activation; HBx aa. 43-154 can only stimulate the activity of HBV enhancer II/core (Fig. 7.3). Consistent with the results in Chapter 3, we also observed that knocking down SREBP-1 abrogated the activation of HBV enhancers by HBx (Fig. 7.3). These results confirm that SREBP-1 is involved in the HBx-induced HBV enhancers' activation. More specifically, HBx aa. 1-127 activates SREBP-1c which is involved in the process of HBV enhancer I/X activation by HBx aa. 1-127; HBx aa. 43-154 enhances the transactivation of SREBP-1a which plays an important role on HBV enhcnaer II/core activation by HBx aa. 43-154 (Fig. 7.3). We then found that HBx activates SREBP-1 is essential for the regulation of HBV replication by HBx (Fig. 7.4 and 7.5).

Taken together, from Chapters 3 to 7, we demonstrated the mechanisms of how HBx regulates HBV replication through SREBP-1-dependent activation of HBV enhancers. Since in Chapter 5, we observed that only nuclear HBx but not cytoplasmic HBx can transactivate SREBP-1a and stimulate HBV enhcancer activity, we assumed that both HBx aa. 1-127 and HBx aa. 43-154 regulates SREBP-1 and HBV enhancers in the nucleus. However, we do not have data to support this now. Further study is required to understand if different subcellular localization of truncated forms of HBx regulates SREBP-1 and HBV enhcancers differentially.

Numerous studies have demonstrated that HCV infection inhibits PTEN through multiple mechanisms [147-149]. However, the effect of PTEN on HCV was not fully understood. In Chapter 9, we first demonstrated that PTEN inhibits HCV entry, PTEN lipid phosphatase activity is required; PTEN has no effect on HCV translation; PTEN inhibits HCV replication and secretion, PTEN protein phosphatase activity is required (Fig. 9.1 to 9.4).

The activity of PTEN can be regulated by protein-protein interaction [45]. HCV core is a structural protein of HCV and functions mainly in the formation of the viral nucleocapsid to protect viral RNA [23]. Therefore, HCV core contains both lipid and RNA binding activities. Moreover, HCV core also interacts with HCV NS5A protein and host proteins [274,299]. Therefore, we assumed that HCV core may interact with PTEN and modulate the phosphatase activity of PTEN. We found that PTEN interacts with HCV core protein. HCV core aa. R50 and PTEN aa. 1-186 are essential for the interaction (Fig. 9.5 to 9.7). Previous studies have demonstrated that expression of HCV core can inhibit PTEN translation by blocking PTEN

mRNA translation in a 3'-UTR dependent manner [147] and activation of NF-κB [149]. Our study displays a new mechanism for how HCV core inhibits PTEN.

Inhibition of PTEN by HCV core may be important for HCV entry, replication and secretion. PTEN suppresses PI3K/Akt pathway through its lipid phosphatase activity [45]. Previous study has reported that HCV infection antivates PI3K/Akt pathway and enhances HCV viral entry, HCV entry receptor CD81 and its co-receptor claudin-1are involved in the process [256]. Therefore, PTEN may inhibit HCV entry through suppression of PI3K/Akt pathway (Fig. 14.1). Loss of PTEN protein phosphatase activity up-regulates HCV secretion through accumulation of large lipid droplets [259]. HCV core recruits HCV NS proteins to lipid droplets, which is essential for formation of the membrous web and HCV replication [300]. Therefore, PTEN may regulate HCV replication and secretion thourhg modulation of lipid metabolism (Fig. 14.1).

When we tried to determine whether the interaction of HCV core and PTEN might affect PTEN phosphatase activity, we could not purify the full-length HCV core protein. Thus, instead of full-length HCV core protein, we purified HCV-2a core domain I and HCV-2a core domain I R50A proteins. To determine the binding affinity between purified HCV 2a core domain I and PTEN proteins, a dose-dependent GST pull-down assay was performed [301,302]. We found that as increasing the concentration of wild type HCV-2a core domain I protein and HCV-2a core domain I R50A protein, more pull-down proteins were detected (Fig. 9.7). Therefore, we conclude that HCV core interacts with PTEN, mutation of R50A leads to weakening the binding affinity. However, we did not detect the interaction between HCV-2a core R50A and PTEN in cells. It may be for two reasons. First, previous studies have determined that PTEN associates with many cellular proteins [45]; therefore, in cells, HCV-2a core may compete with other proteins to interact with PTEN. Second, when the input concentration of HCV 2a core domain I R50A protein was very low, it was hardly detected by Western blotting assay (Fig. 9.7). Therefore, R50A mutant of HCV core weakens the binding affinity of PTEN and may result in reducing the amount of PTEN-interacted HCV-2a core R50A protein to an undetectable level. When we determined the effect of HCV core domain I on PTEN PIP_3 phosphatase activity, we observed that HCV core domain I increased PTEN PIP_3 phosphatase activity in a dose-dependent manner (Fig. 9.7E). As shown in Fig. 9.2, PTEN lipid phosphatase activity inhibits HCV entry. These data imply that HCV-2a core interacts with PTEN may inhibit HCV entry in a dose-

dependent manner. It is possible that a primary HCV infection followed by a secondary reinfection with a distinct HCV at alater time point, this process is called HCV superinfection [303]. Our data may indicate HCV-2a may inhibit HCV superinfection through modulation of PTEN lipid phosphatase activity in a dose dependent manner. We just used HCV-2a core in the PTEN phosphatase assay experiment. In the future, we want to determine if other genotypes of HCV core interact with PTEN and regulate PTEN phosphatase activities.

After demonstrating the effect of PTEN on HCV infection, we were then interested in characterizing whether PTEN-Long, an isoform of PTEN, may regulate HCV infection as well as PTEN. We first found that intracellular PTEN-Long inhibits HCV replication as effectively as intracellular PTEN (Fig. 11.1). Since PTEN-Long allows secretion in exosomes and function outside of the cell [59,60], we were then interested in studying whether extracelluar PTEN-Long may regulate HCV replication. We observed that extracellular PTEN-Long, but not PTEN, can enter the HCV replicating cells and inhibit HCV replication (Fig. 11.2). Moreover, the interaction of HCV core and PTEN-Long is essential for the inhibition of HCV replication by PTEN-Long (Fig. 11.3). This finding indicates that PTEN-Long treatment may be an option for HCV therapy.

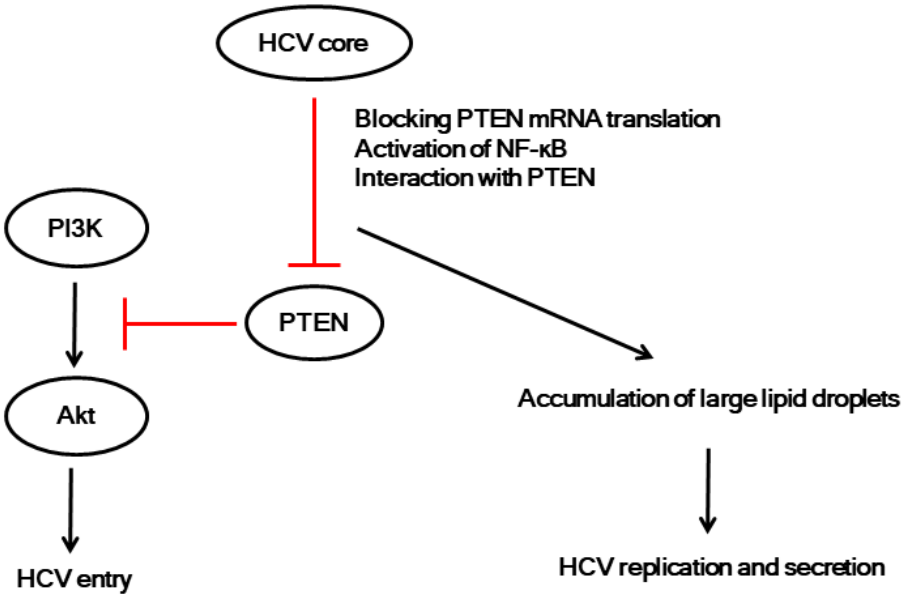

Fig. 14.1. Potential mechanisms of how HCV core regulates HCV life cycle through inhibition of PTEN.

We proposed studying HBV HCV co-infection using four models. To study the acute HBV HCV co-infection, we planded to co-transfect both HBV genomic DNA and HCV genomic HCV in Huh-7 cells; to study whether HCV affects the early stage of HBV infection, we planned to transfect HBV genomic DNA into HCV-replicating Huh-7 cells; to study whether HBV regulates the early stage of HCV infection, we wanted to transfect HCV RNA into HCV-replicating Huh-7 cells; to study whether HBV and HCV interfere with each other in chronic HBV HCV co-infection, we planned to establish the HBV and HCV co-replicating Huh-7 cells. In Chapter 13, we showed that HBV and HCV do not interfere with each other in HBV genome plasmid transfected HCV-replicating Huh-7 cells (Fig. 13.1); HBV enhances the early stage of HCV infection in HCV RNA transfected HBV-replicating Huh-7 cells (Fig. 13.7). We will study other two models of HBV HCV co-infection in the future study.

PTEN negatively regulates the activity of SREBP-1 through suppression of the PI3K/Akt pathway [45,80]. HBx can also inhibit PTEN through different mechanisms. Previous studies have demonstrated that HBx inhibits the function of p53 [285] and therefore increases alpha-fetoprotein (AFP) expression [286]. AFP associates with PTEN in the cytoplasm and reduces PTEN level [287]. MicroRNAs are a family member of small non-coding nucleotide RNA molecules which translationally regulate protein expression through targeting messenger RNAs (mRNAs). This process results in cleavage of the mRNA, inhibition of translation initiation and shortening the poly(A) tail of mRNA [304]. Previous studies have reported that HBx up-regulates miR-21, miR-29a, miR-145 and miR-222 which target the 3'-UTR of PTEN mRNA and inhibit PTEN protein expression [289,290,305]. Several studies have demonstrated that HBx can activate PI3K/Akt pathway [306-309]. Recent studies from Dr. Mengsen Li's group have reported that HBx stimulates AFP expression and AFP interacts and inhibits PTEN [291]. Moreover, this group has also reported that HBx enhances AFP expression and thus PI3K/Akt/mTORC1 pathway [291,310]. Activation of Akt/mTORC1 pathway leads to activate SREBP-1 [81,82]. As shown in Fig. 13.4 and 13.5, we observed that modulating the PTEN/SREBP-1 pathway can alter HBV replication in either HBV mono-infected cells or HBV HCV co-infected cells. Reduced PTEN levels upon HBx overexpression increased the protein level of nuclear SREBP-1 in Huh-7 cells (Fig. 13.6A). These data indicate the possible mechanisms of how HBx regulates HBV replication. HBx modulates PTEN/PI3K/Akt/mOTRC1/SREBP-1 pathway. In Chapters 3-7, we found that HBx enhances the

activation of SREBP-1 and thus stimules HBV replication. Therefore, the regulation of PTEN/SREBP-1 pathway by HBx is important for stimulating HBV replication (Fig. 14.2). Interestingly, HBx can up-regulates mTORC1/S6K pathway though a PI3K/Akt independent manner. HBx activates IKKβ which leads to inhibit TSC1 and up-regulate mTORC1/S6K pathway [311]. It is a potential mechanism of how HBx activates SREBP-1 and thus enhances HBV replication (Fig. 14.2).

Unexpectedly, we observed that the HBx and HCV core cannot additively reduce PTEN protein level or increase the nuclear SREBP-1 protein level in HCV core-expressing Huh-7 cells transfected with HBx expressing plasmid (Fig. 13.6). However, this is just one model to study how the HBx and HCV core regulate PTEN and SREBP-1. In this model, the data may indicate that HBV superinfection in CHC do not additively modulate PTEN/SREBP-1 pathway. Further work is required to determine how the HBx and HCV core modulate PTEN and SREBP-1 in HCV superinfection in CHB or HBV HCV chornic co-infection. Therefore, it is necessary to repeat this experiment in HBx-expressing Huh-7 cells transfected with HCV core and HBx and HCV core stably expressing cells. In Chapter 11, we showed that PTEN-Long treatment inhibts HCV replication (Fig. 11.2). In Chapter 13, we demonstrated that overexpression of PTEN inhibits HBV replication (Fig. 13.2). However, the role of PTEN-Long in HBV replication has not been studied. Therefore, the effect of PTEN-Long on HBV and HCV replications in HBV or HCV mono-infections and HBV HCV co-infection is worthy of study in the future.

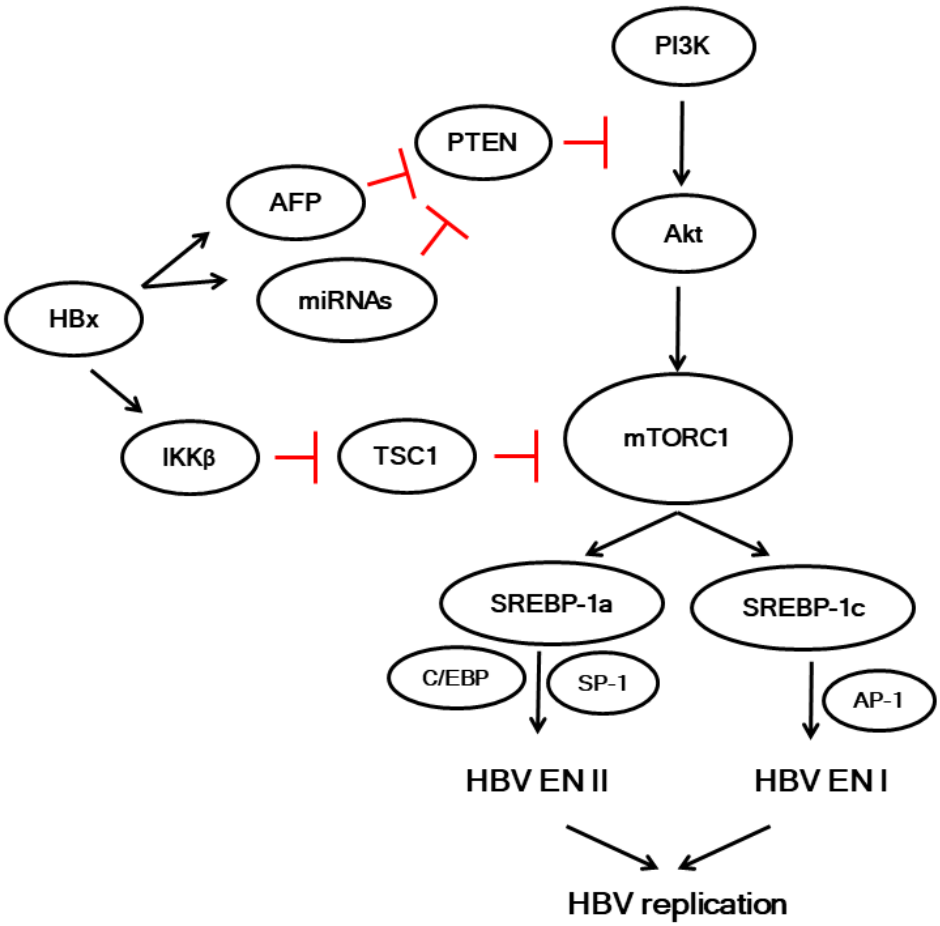

Fig. 14.2. Potential mechanisms of how HBx modulates PTEN/SREBP-1 pathway and enhances HBV replication.

14.2 Conclusion

- HBx activates SREBP-1 activity at the transcription level which is involved in up-regulation of HBV enhancers and thus stimulation of HBV replication. Expression of HBx stimulates SREBP-1a and FANS transcription activation and induces intracellular lipid accumulation and cell proliferation. This effect may partially explain how HBV infection causes the development of steatosis and enhances of the risk factor of HCC.
- PTEN inhibits HCV entry, replication and secretion. However, PTEN has a little effect on HCV RNA translation. PTEN interacts with HCV core protein. HCV core aa. R50 and PTEN aa. 1-186 are essential for the interaction. The interaction between PTEN and HCV core inhibits PTEN levels which enhances HCV replication. These findings may explain the mechanisms of how HCV infection inhibits the tumor suppresor, PTEN, and thus enhances the risk factor of HCC.
- PTEN-Long treatment inhibits HCV replication in a dose-dependent manner. This finding indicates that PTEN-Long treatment may contribute to HCV therapy.
- Modulation of PTEN/SREBP-1 pathway alters the HBx-mediated HBV replication. HCV replication is stimulated by PTEN knockdown and is modestly inhibited by SREBP-1 knockdown. HBx and HCV core do not additively decrease PTEN protein level and enhance SREBP-1 activation. PTEN and SREBP-1 play the same roles in regulation of HBV or HCV replication in either HBV and HCV mono-infections or HBV HCV co-infection. Our findings suggest that HBx does not alter HCV infection nor does the HCV core affect HBV infection in HBV HCV co-infection.

14.3 Future experiments

- In Chapter 5, we observed that only nuclear HBx but not cytoplasmic HBx can transactivate SREBP-1a and stimulate HBV enhcancer activity. In Chapter 7, we established the role of HBx aa. 1-127 and HBx aa. 43-154 in the regulation of SREBP-1, HBV enhancers and HBV replication. However, how different subcellular localization of

truncated forms of HBx regulates SREBP-1 and HBV enhcancers is still not clear. We will determine the subcellular localization of truncated forms of HBx. Then we will determine whether nuclear or cytoplasmic truncated forms of HBx regulate SREBP-1 and HBV enhancers differentially.

- We plan to study the mechanisms of how HBx regulates PTEN/SREBP-1 in the future. Previous studies have established several indirect mechanisms as to how HBV inhibits PTEN; however, whether HBx directly interacts with PTEN is still unknown. As shown in Chapter 7, different truncated forms of HBx differentially regulate the activation of SREBP-1a and SREBP-1c. However, which domain of HBx is required for regulation of PTEN is still not clear. Future research will clarify these issues.
- A previous study has established that HCV core R50 is required for interaction of HCV core with NS5A protein [264]. Previous studies have demonstrated that NS5A can inhibit PTEN thorugh activation of NF-κB [154-157]. No study has reported whether NS5A directly interacts with PTEN. However, it is possible that the HCV core interacts with both NS5A and PTEN and thus these three proteins may form a trimer in HCV-infected cells. As shown in Fig. 9.7, we observed that the HCV core interacts with PTEN and reduces the protein level of PTEN. Therefore, future study will be needed to understand whether HCV core and NS5A synergistically regulate PTEN through froming the trimer. Future research is required to establish whether HCV core, NS5A and PTEN can form a trimer and whether this process alters PTEN functions and HCV life cycle.
- Interactions between the HBV and HCV have been difficult to study because of the lack of appropriate co-infection model systems.Therefore, it is important to establish HBV HCV co-replicating cell lines. In Chapter 13, we only study HBV HCV co-infection in HBV genome plasmid transfected HCV genomic RNA replicating cells and HBV RNA transfected HBV replicating cells. Future research is required to determine the role of the PTEN/SREBP-1 pathway in HBV and HCV life cycle in HBV HCV co-replicating cells.
- In Chapter 11, we have established that PTEN-Long treatement can inhibit HCV replication. However, whether PTEN-Long treatment regulate HBV replication is still not clear. Therefore, future work is required to establish whether PTEN-Long treatment inhibits HBV replication in HBV mono-infection and the effect of PTEN-Long treatment on HBV and HCV replication in HBV HCV co-infection.

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